

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371**

U.S. APPLICATION NO. (If known, sect. 37 C.F.R. 1.5)

09/743533**"EXPRESS MAIL" MAILING CERTIFICATION**Number: **EL659498785US** Date of Deposit: **January 10, 2001**

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, BOX PCT, Washington, D.C. 20231, on January 10, 2001.

Typed or Printed Name: Vincent DiazSigned: Vincent M. Diaz

INTERNATIONAL APPLICATION NO.

PCT/AU99/00563

INTERNATIONAL FILING DATE

12 July 1999

PRIORITY DATE CLAIMED

10 July 1998

TITLE OF INVENTION

MODIFIED PROTEINS

APPLICANT(S) FOR DO/EO/US

(1) Rudi APPELS, Aranda, Australia; (2) Matthew MORELL, Aranda, Australia;
(3) Frank BEKES, Beecroft, Australia; and (4) Laszlo TAMAS, Budapest, Hungary

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information.

Amendment (under Article 34) and substitute pages 3, 30, 43 - 45
International Preliminary Examination Report
Preliminary Amendment with appended Claims for Examination

09/743533

17. ☒ The following fees are submitted:**Basic National Fee (37 CFR 1.492(a)(1)-(5)):**

Search Report has been prepared by the EPO or JPO \$860.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)
..... \$690.00

No international preliminary examination fee paid to USPTO (37 CFR 1.482)
but international search fee paid to USPTO (37 CFR 1.445(a)(2)) \$710.00

Neither international preliminary examination fee (37 CFR 1.482) nor international search
fee (37 CFR 1.445(a)(2)) paid to USPTO \$1000.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)
and all claims satisfied provisions of PCT Article 33(2)-(4) \$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =**CALCULATIONS** PTO USE ONLY

\$ 1,000.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30
months from the earliest claimed priority date (37 CFR 1.492(e)).

\$ 130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total Claims	27	-20 =	7
Independent Claims	3	-3 =	0
Multiple dependent claims (if applicable)			

+ \$ 270.00

TOTAL OF ABOVE CALCULATIONS =

\$ 1,256.00

Reduction by 1/2 for filing by small entity, if applicable. Verified Small
Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).

\$

SUBTOTAL =

\$ 1,256.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30
months from the earliest claimed priority date (37 CFR 1.492(f)).

\$

+

TOTAL NATIONAL FEE =

\$ 1,256.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property

\$

TOTAL FEES ENCLOSED =

1,256.00

Amount to be:
refunded \$

charged \$

a. ☒ A check in the amount of \$1,256.00 to cover the above fees is enclosed.b. ☐ Please charge my Deposit Account No. 06-1300 in the amount of \$ to cover the above fees.c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1300 (Order No. A-70233/RFT).

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Richard F. Trecartin, Esq.
FLEHR HOHBACH TEST
ALBRITTON & HERBERT LLP
Suite 3400
Four Embarcadero Center
San Francisco, California 94111-4187
Telephone: (415) 781-1989

SIGNATURE

Richard F. Trecartin
NAME31,801
REGISTRATION NUMBER

09/743533

PATENT

Attorney Docket No.: A-70233/RFT

JC07 Rec'd PCT/PTO

10 JAN 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE*In re* application of:APPELS, *et al.***NATIONAL PHASE ENTRY OF:
PCT/AU99/00563**

Serial No.: Not Yet Assigned

Filed: Herewith

For: MODIFIED PROTEINS

**EXPRESS MAIL CERTIFICATION
UNDER 37 CFR §1.10**Express Mail No. EL659498785USDate January 10, 2001

I hereby certify that this document and any documents listed and referred to as enclosed herein are being deposited with the United States Postal Service on the date given above in an envelope bearing "Express Mail Post Office To Addressee" and addressed to: Assistant Commissioner for Patents **Box PCT**, Washington, D.C. 20231.

Typed Name: VINCENT DIAZSigned: Vincent M. Diaz**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents

BOX PCT

Washington, DC 20231

Sir:

Prior to examination, please make the following amendments.

IN THE CLAIMS:

8. (Amended) The method according to [any one of claims 1 to 7] claim 1 wherein the glutenin or seed-storage protein is selected from the group consisting of low molecular weight glutenins, high molecular weight glutenins, gliadins, puroindolines, grain softness proteins, friabilins, and Chloroform/Methanol-soluble proteins.

10. (Amended) A modified glutenin or seed-storage protein having an ability to incorporate into gluten and having an exogenous amino acid domain inserted therein which confers to the modified protein the ability to bind a ligand or other macromolecule produced by the method according to [any one of claims 1 to 9] claim 9.

Serial No.: Not Assigned
Filed: HERewith

15. (Amended) A modified glutenin or seed-storage protein according to [any one of claims 10 to 14] claim 11 wherein the glutenin or seed-storage protein is selected from the group consisting of low molecular weight glutenins, high molecular weight glutenins, gliadins, puroindolines, grain softness proteins, friabilins, and Chloroform/Methanol-soluble proteins.

17. (Amended) An isolated nucleic acid molecule encoding a modified glutenin or seed-storage protein according to [any one of claims 10 to 16] claim 11.

23. (Amended) Use of a modified glutenin or seed-storage protein according to [any one of claims 10 to 16] claim 11 in the preparation of a food product.

25. (Amended) Use of a modified glutenin or seed-storage protein according to [any one of claims 10 to 16] claim 11 in the preparation of a non-food product.

27. (Amended) Use of a grain or part of a grain containing a modified glutenin or seed-storage protein according to [any one of claims 10 to 16] claim 11 in the preparation of a food product.

Serial No.: Not Assigned
Filed: HEREWITH

REMARKS

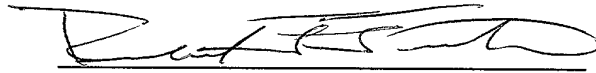
Applicant filed an Article 34 amendment with the International Preliminary Examining Authority, Australia, on May 31, 2000 in response to the Written Opinion. A copy of that amendment is being filed herewith. Applicant has amended claims 8, 10, 15, 17, 23, 25 and 27 of the claims submitted in that amendment to correct claiming multiple dependency. A copy of the claims to be examined in this application are appended hereto.

Respectfully submitted,

FLEHR HOHBACH TEST
ALBRITTON & HERBERT LLP

Date

Jan 10, 2001



Richard F. Trecartin, Reg. No. 31,801

Four Embarcadero Center, Suite 3400
San Francisco, California 94111
Telephone: (415) 781-1989

Serial No.: Not Assigned
Filed: HERewith

CLAIMS AS AMENDED FOR EXAMINATION

1. A method of producing a modified glutenin or seed-storage protein, the method comprising adding to the protein an exogenous amino acid domain which confers to the modified protein the ability to bind a ligand or other macromolecule, wherein the modified protein has an ability to incorporate into gluten.
2. The method according to claim 1 wherein the domain includes one or more cysteine residues.
3. The method according to claim 1 wherein one or more cysteine residues are incorporated at one or both ends of the amino acid sequence of the protein.
4. The method according to claim 1 wherein the modified protein further comprises one or more exogenous amino acid residues which confer to the protein an enhanced ability to incorporate into gluten.
5. The method according to claim 1 wherein the ligand or other macromolecule to which the domain binds is selected from the group consisting of lipid and starch.
6. The method according to claim 5 wherein the domain capable of binding lipid is derived from barley oleosin gene or the lipid-binding regions of wheat CM16 protein.
7. The method according to claim 5 wherein the domain capable of binding starch is derived from glucoamylase from *Aspergillus niger*.

Serial No.: Not Assigned
Filed: HEREWITH

8. The method according to claim 1 wherein the glutenin or seed-storage protein is selected from the group consisting of low molecular weight glutenins, high molecular weight glutenins, gliadins, puroindolines, grain softness proteins, friabilins, and Chloroform/Methanol-soluble proteins.

9. The method according to claim 8 wherein the glutenin or seed-storage protein is C hordein from barley.

10. A modified glutenin or seed-storage protein having an ability to incorporate into gluten and having an exogenous amino acid domain inserted therein which confers to the modified protein the ability to bind a ligand or other macromolecule produced by the method according to claim 1.

11. A modified glutenin or seed-storage protein having an ability to incorporate into gluten and having an exogenous amino acid domain inserted therein which confers to the modified protein the ability to bind a ligand or other macromolecule.

12. A modified glutenin or seed-storage protein according to claim 11 wherein the ligand or other macromolecule to which the domain binds is selected from the group consisting of lipid, and starch.

13. A modified glutenin or seed-storage protein according to claim 12 wherein the domain capable of binding lipid is derived from barley oleosin gene or the lipid-binding regions of wheat CM16 protein.

14. A modified glutenin or seed-storage protein according to claim 12 wherein the domain capable of binding starch is derived from glucoamylase from *Aspergillus niger*.

Serial No.: Not Assigned
Filed: HEREWITH

15. A modified glutenin or seed-storage protein according to claim 11 wherein the glutenin or seed-storage protein is selected from the group consisting of low molecular weight glutenins, high molecular weight glutenins, gliadins, puroindolines, grain softness proteins, friabilins, and Chloroform/Methanol-soluble proteins.

16. A modified glutenin or seed-storage protein selected from the group consisting of ANG/SDB/Cys7Cys 236, ANG/OHBD/Cys7Cys236, and ANG/CM16/Cys7Cys126, as hereinbefore defined.

17. An isolated nucleic acid molecule encoding a modified glutenin or seed-storage protein according to claim 11.

18. A cell containing an isolated nucleic acid molecule according to claim 17 such that an expression of the nucleic acid molecule, the cell produces the modified glutenin or seed-storage protein.

19. The cell according to claim 18 selected from the group consisting of bacteria, yeast, plant, insect and mammal.

20. The cell according to claim 19 being *Escherichia coli*.

21. The cell according to claim 19 being *Pichia sp.* or *Saccharomyces cerevisiae*.

22. The cell according to claim 19 being a recombinant wheat cell.

23. Use of a modified glutenin or seed-storage protein according to claim 11 in the preparation of a food product.

Serial No.: Not Assigned
Filed: HERewith

24. The use according to claim 23 wherein the food product is selected from the group consisting of leavened or unleavened breads, pasta, noodles, breakfast cereals, snack foods, cakes, pastries, and foods containing flour-based sauces.

25. Use of a modified glutenin or seed-storage protein according to claim 11 in the preparation of a non-food product.

26. The use according to claim 25 wherein the non-food product is selected from the group consisting of films, coatings, adhesives, building materials, and packaging materials.

27. Use of a grain or part of a grain containing a modified glutenin or seed-storage protein according to claim 11.

PATENT
Attorney Docket No.: A-70233/RFT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

APPELS et al.

Serial No. 09/743,533

Filed: January 10, 2001

For: MODIFIED PROTEINS

Examiner: Not Yet Assigned

Group Art Unit: Not Yet Assigned

CERTIFICATE OF MAILING

I hereby certify that this correspondence, including listed enclosures, is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, DC 20231 on: October 1, 2001.

By: Wendy Wilson

Wendy Wilson

AMENDMENT RE SEQUENCE LISTING

BOX SEQUENCE LISTING

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

The present Preliminary Amendment is in response to the Notice to Comply mailed April 30, 2001 and is submitted to comply with requirements for patent applications containing nucleotide sequence and/or amino acid sequence disclosures in adherence with rules 37 C.F.R. § 1.821-1.825.

The amendment is submitted within the statutory period, making this a timely response. While Applicants believe that no fees are due at this time, the Commissioner is authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 06-1300 (Our Order No. A-70233/RFT).

Please amend the application as follows and to comply with requirements for patent applications containing nucleotide sequence and/or amino acid sequence disclosures in adherence with rules 37 C.F.R. § 1.821-1.825 as follows:

Serial No.: 09/743,533

Filed: January 10, 2001

IN THE SPECIFICATION:

Please replace the Sequences Listing starting on page 31 with the enclosed Sequence Listing. Delete the Sequence Listing numbered pages 1-11 at the end of the specification.


REMARKS

These amendments are made in adherence with 37 C.F.R. § 1.821-1.825. This amendment is accompanied by a floppy disc containing the above named sequence, SEQUENCE ID NUMBERS 1-26, in computer readable form, and a paper copy of the sequence information. The information contained in the computer readable disk is identical to that of the paper copy. This amendment contains no new matter. Applicant submits that this amendment, the accompanying computer readable sequence listing, and the paper copy thereof serve to place this application in a condition of adherence to the rules 37 C.F.R. § 1.821-1.825. Entry of this amendment is respectfully requested. The amendments are made in adherence with 37 C.F.R. § 1.821-1.825.

Respectfully submitted,

FLEHR HOHBACH TEST
ALBRITTON & HERBERT LLP

Dated: October 1, 2001


Richard F. Trecartin, Reg. No. 31,801

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SEQUENCE LISTING

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65				70					75				80		
Gly	Cys	Pro	Arg	Glu	Val	Gln	Met	Asp	Phe	Val	Arg	Ile	Leu	Val	Thr
			85					90				95			
Pro	Gly	Tyr	Cys	Asn	Leu	Thr	Thr	Val	His	Asn	Thr	Pro	Tyr	Cys	Leu
			100					105				110			
Ala	Met	Glu	Glu	Ser	Gln	Trp	Ser	Cys	Ile	Trp					
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			20					25					30		
Glu	Met	Pro	Gly	Pro	Pro	Tyr	Leu	Ala	Lys	Gln	Glu	Cys	Cys	Glu	Gln
			35				40					45			
Leu	Ala	Asn	Ile	Pro	Gln	Gln	Cys	Arg	Cys	Gln	Ala	Leu	Arg	Tyr	Phe
	50					55					60				
Met	Gly	Pro	Lys	Ser	Arg	Pro	Asp	Gln	Ser	Gly	Leu	Met	Glu	Leu	Pro
65					70					75				80	
Gly	Cys	Pro	Arg	Glu	Val	Gln	Met	Asn	Phe	Val	Pro	Ile	Leu	Val	Thr
				85					90				95		
Pro	Gly	Tyr	Cys	Asn	Leu	Thr	Thr	Val	His	Asn	Thr	Pro	Tyr	Cys	Leu
			100					105					110		
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<400> 25

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<400> 26

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Thr	Lys	Leu	Asn	Ser	Cys	Arg	Asn	Tyr	Leu	Leu	Asp	Arg	Cys	Ser	Thr
			20					25					30		
Met	Lys	Asp	Phe	Pro	Val	Thr	Trp	Arg	Trp	Trp	Lys	Trp	Trp	Lys	Gly
		35					40				45				
Gly	Cys	Gln	Glu	Leu	Leu	Gly	Glu	Cys	Cys	Ser	Arg	Leu	Gly	Gln	Met
	50					55					60				
Pro	Pro	Gln	Cys	Arg	Cys	Asn	Ile	Ile	Gln	Gly	Ser	Ile	Gln	Gly	Asp
65				70					75					80	
Leu	Gly	Gly	Ile	Phe	Gly	Phe	Gln	Arg	Asp	Arg	Ala	Ser	Lys	Val	Ile
			85					90					95		
Gln	Glu	Ala	Lys	Asn	Leu	Pro	Pro	Arg	Cys	Asn	Gln	Gly	Pro	Pro	Cys
		100						105					110		
Asn	Ile	Pro	Gly	Thr	Ile	Gly	Tyr	Tyr	Trp	Cys	Ile	Trp			
		115					120					125			

Modified ProteinsTechnical Field

The present invention is directed generally to producing modified proteins and particularly to producing modified glutenin or seed-storage proteins.

Background Art

Wheat storage proteins are classified on the basis of their solubility into two classes. The gliadins are readily soluble in aqueous alcohols and are monomeric proteins with only intramolecular disulphide bonds. The glutenins are present in high molecular weight polymers, stabilised by intermolecular disulfide bonds and are not soluble in aqueous alcohols without reducing agent (Kasarda 1989). These proteins are present in high amount in the endosperm and are considered to act as a store of nitrogen, carbon and sulphur for seed germination.

Glutenins form a continuous proteinaceous network called gluten. The unique physico-chemical properties of gluten determine the ability of wheat dough to be processed into baked goods (bread, biscuits, cakes), pasta noodles and other food products. It is understood that the glutenins, which form crosslinks with each other through disulfide bonds, are the most important molecules producing the viscoelastic properties of wheat flour dough (MacRitchie 1992). The unique position of wheat in bread making is due to the ability of the dough to retain gas on expansion. The gluten accounts for about 10% of the dough, and consists mainly of proteins (70-80%) together with starch and lipids. Starch could be granular and damaged starch. The lipid reserves of wheat are non-polar, structural and endosperm lipids (Gan et al., 1995). Structural lipids are also called polar lipids. The endosperm lipids are divided into non-starch lipids and starch lysophospholipids. The structure and properties of gluten are determined by molecular interactions and it is important that these be understood if the functional properties of gluten are to be manipulated.

A dough results from a large variety of interactions between flour constituents facilitated by water. Starch takes up about 46% of the water and damaged starch contributes significantly to the water absorption. It has been shown that during hydration, proteins exude visible strands or fibrils. Specific proteins of flour are bound to flour lipids (polar) upon addition of water (Morrison 1989).

Dough development is visualised as a re-orientation of glutenin polymers to form a membrane network with viscoelasticity and gas retaining properties. Covalent (disulfide) and noncovalent (hydrogen, hydrophobic and ionic) bonds are involved in formation of a fully developed dough.

5 Interactions are further modified during fermentation, baking and even after baking. The disulphide bonds of flour proteins play a key role in the interactions in dough. The bonds form relatively strong crosslinks within and between polypeptide chains and also stabilise other less energetic bonds. Disulphide bonds provide the required stability for the protein matrix until

10 the loaf structure is set by the gelatinisation of the starch and the thermal denaturation of the proteins during baking. Hydrogen bonds are considerably weaker than covalent bonds, but contribute significantly to the structure of dough. A unique feature is the ability to interchange with other hydrogen bonds, which facilitate reorientation of protein chains and allow for stress

15 relaxation. Hydrophobic bonds result from nonpolar groups of flour constituents. Because these bonds are reversible, they can readily accommodate viscous flow and thereby facilitate mechanical dough development. Ionic bonds play relatively small part in dough structure formation but some specific components have an ionisable part or parts.

20 Therefore ionic bond interactions could be important for the rheological properties (for a review, see Bushuk 1998).

A major limitation to evaluating the contributions of various groups of proteins, and of specific structural features of these molecules, to dough functionality has been the lack of appropriate systems that allow specific

25 proteins to be incorporated and tested within the dough. The situation has recently changed, however, due to two advances. The first is the development of small scale testing equipment (Mixograph, Extensograph) with appropriate procedures for incorporating exogenous proteins, including polymeric glutenins into the dough (Bekes et al., 1994). Advantages of this

30 system are the small amount of proteins required for test and the ability to rapidly test multiple samples produced by, for example, protein engineering. The second recent advance is the development of a reliable transformation system for wheat (Weeks et al., 1993, Witrzens et al., 1998), which allows the modification of storage protein composition by the expression of new

35 proteins with, for instance, designed characteristics.

ART 34 AMDT

3

To alter protein-protein, protein-lipid and protein-starch interactions within the gluten matrix, the present inventors have developed a system which enables the incorporation of new surface active molecules or parts of molecules into the gluten matrix.

5 Disclosure of Invention

In a first aspect, the present invention consists in a method for producing a modified glutenin or seed-storage protein, the method comprising adding to the protein an exogenous amino acid domain which confers to the modified protein the ability to bind a ligand or other
10 macromolecule, wherein the modified protein has an ability to incorporate into gluten.

In one preferred embodiment, the modified glutenin or seed-storage protein further contains one or more exogenous amino acid residues added to its amino acid sequence. More preferably, the one or more amino acid
15 residues are one or more cysteine residues. Preferably, the one or more cysteine residues are incorporated at one or both ends of the amino acid sequence of the protein. The addition of the one or more cysteines allows the modified proteins to be more easily incorporated into gluten in use. The further modifications to the glutenin or seed-storage proteins produced
20 according to the present invention allow or can assist in the incorporation of that protein into the gluten network for food or industrial use.

The present inventors have found that incorporating exogenous amino acid sequences (domains) from proteins other than glutenins or seed storage proteins into glutenin or seed-storage proteins modifies the general properties
25 of gluten, particularly when the modified proteins are used in a range of food applications.

Figure 16 provides a schematic of the scheme for identifying transgenes in transformed wheat plants by polymerase chain reaction (PCR). A primer pair straddling the interface between the gene and its promoter
30 (from the gene for the high molecular weight glutenin Bx17 for example) ensures that no false positives are detected arising from the high homology between C hordein and gliadin genes.

The binding domain can be any domain that will bind ligands that may be useful in food preparation or in food compositions. In a preferred form,
35 the binding domain is a ligand capable of binding lipids or starches. The

present inventors have found that the lipid-binding domain of the barley oleosin gene, the lipid-binding regions of the wheat CM16 protein, and the starch-binding domain of the glucoamylase from *Aspergillus niger* are particularly suitable for the present invention. It will be appreciated, however, that other natural or modified domains would also be suitable for the present invention.

One glutenin or seed-storage protein that has been modified by the present inventors is the C hordein gene from barley. It will be appreciated, however, that other glutenin or seed-storage proteins may also be modified according to the present invention. In wheat, such glutenin or seed proteins include low molecular weight glutenins, high molecular weight glutenins, gliadins, puroindolines or grain softness proteins (also known as friabilins), or Chloroform/Methanol-soluble proteins. Homologues of these proteins exist in other cereals such as diploid, tetraploid and hexaploid wheats, rye, triticale, barley, oats, rice, sorghum, millet and maize and the genes encoding these proteins may also be modified according to the present invention.

In a second aspect, the present invention consists in a modified glutenin or seed-storage protein having a domain inserted therein which confers to the protein the ability to incorporate into gluten or bind a ligand or other macromolecule.

In one preferred embodiment, the modified glutenin or seed-storage protein is produced by the method according to the first aspect of the present invention.

In another preferred embodiment, the modified glutenin or seed-storage protein is ANG/SBD/Cys7Cys236, ANG/OHBD/Cys7Cys236 or ANG/CM16/Cys7Cys236.

In a third aspect, the present invention consists in an isolated nucleic acid molecule encoding a modified glutenin or seed-storage protein according to the second aspect of the present invention.

In a fourth aspect, the present invention consists in an isolated nucleic acid molecule according to the third aspect of the present invention incorporated into a cell such that on expression of the nucleic acid molecule, the cell produces the modified glutenin or seed-storage protein.

The cell may be a recombinant bacterial cell, for example, which is capable of producing the modified glutenin or seed-storage protein. Preferably the bacterial cell is *Escherichia coli*. Alternatively, the cell may be

a yeast such as *Pichia* sp. or *Saccharomyces cerevisiae*, an insect cell using an expression system such as the baculovirus expression system, or a mammalian cell. Alternatively, the cell may be a plant cell of a recombinant plant which is capable of producing the modified glutenin or seed-storage protein in the plant's seeds. Preferably the plant cell is a recombinant wheat cell.

In fifth aspect, the present invention consists in the use of a modified glutenin or seed-storage protein according to the second aspect of the present invention in the preparation of a food product.

Examples of food products include leavened or unleavened breads, pasta, noodles, breakfast cereals, snack foods, cakes, pastries or other foods containing flour-based sauces or ingredients.

The modified glutenin or seed-storage proteins according to the present invention, in use, are capable of modifying the structure of doughs and other materials containing gluten in ways which add value and utility to the resultant product. The modified glutenin or seed-storage proteins are suitable for use in the food industry as modifiers of food properties.

The present inventors have shown that modified proteins according to the present invention can be produced in bacterial fermentation and that large scale production of the proteins for commercial use is possible.

In sixth aspect, the present invention consists in the use of a modified glutenin or seed-storage protein according to the second aspect of the present invention in the preparation of a non-food product.

Examples of non-food products include, but not limited to, films, coatings, adhesives, building materials or packaging materials. It will be appreciated that the modified proteins according to the present invention would have the same non-food uses as for normal glutenin or seed-storage proteins.

In a seventh aspect, the present invention consists in the use of a grain or part of a grain containing a modified glutenin or seed-storage protein according to the second aspect of the present invention in the preparation of a food product.

It will be appreciated that the modified glutenin or seed-storage proteins according to the present invention may be contained in, or produced by, a transgenic plant produced by the fourth aspect of the present invention. Thus, the grain or other plant products produced by these plants may be used

in the crude form of flour, semolina, bran, pollard, germ fraction, or the like. Such plants may produce a mixture of normal and modified glutenin or seed-storage proteins which can also be used. Mixtures of modified glutenin or seed-storage proteins and other plant-based materials can also be prepared to form improved gluten.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following examples and drawings.

Brief Description of Drawings

Figure 1: Sequence of the pJANGΔCys7Cys236 vector (SEQ ID NO: 14 and SEQ ID NO: 15). Restriction sites and enzymes of the MCS of pJKKm and pGEM-T plasmids are written in bold. Cloning sites for gene insertion into ANG are underlined and written in bold. Cleavage sites for sub-cloning the gene into pET 11d expression vector are underlined.

Figure 2: Nucleotide (SEQ ID NO: 16) and amino acid sequence (SEQ ID NO: 17) of Oleosin Hydrophobic Binding Domain (OHBD). Arrowheads are indicating the direction of primer extension. Smaller letters are indicating extra nucleotides for cloning and amino acids from ANG molecule. Amino acid sequence of the central anti-parallel domain is designated by two arrows.

Figure 3: Nucleotide (SEQ ID NO: 18) and amino acid sequence (SEQ ID NO: 19) of ANGΔCys7Cys236 (molecular mass 18.5 kDa) Cysteine residues are marked with bold letters. Extra two amino acids and six nucleotides are written with smaller letters.

Figure 4: Nucleotide (SEQ ID NO: 20) and amino acid sequence (SEQ ID NO: 21) of the starch binding domain of 1,4-α-D-glucan glucohydrolase from *Aspergillus niger* (molecular mass 11.9 kDa). Small letters are indicating extra nucleotides for cloning and amino acids from ANG molecule. Restriction sites are in bold and underlined.

Figure 5: Nucleotide (SEQ ID NO: 22) and amino acid sequences of CM16 (SEQ ID NO: 23) and CM17 (SEQ ID NO: 24) (molecular mass is 13.4

kDa). Small letters are indicating extra nucleotides for cloning and amino acids from ANG molecule. Restriction sites are in bold and underlined. First amino acid sequence under the nucleotide sequence represents the CM16 protein, while only differences are shown in CM17 protein.

Figure 6: Nucleotide (SEQ ID NO: 25) and amino acid sequence (SEQ ID NO: 26) of Puroindoline A (molecular mass is 14.3 kDa). Small letters are indicating extra nucleotides for cloning and amino acids from ANG molecule. Restriction sites are in bold and underlined.

Figure 7: Expression of the ANG/OHBD/Cys7Cys236 gene. SDS-PAGE analysis followed by Coomassie blue staining. Lane A contains standard molecular weight markers. Lane B shows a control in which the host *E. coli* cell contains the pET-11d vector. Lane C shows the expression of the recombinant ANG/OHBD/Cys7Cys236 protein (migrating slightly further than the 30 kDa marker) from pET-11d containing the ANG/OHBD/Cys7Cys236 gene.

Figure 8: Expression of the ANG/OHBD/Cys7Cys236 gene. SDS-PAGE analysis followed by Western blot using antibodies to C-hordein. Lane A contained standard molecular mass markers. Lane B contained extract of an *E. coli* cell expressing a modified C-hordein gene containing a single cysteine in the N-terminal region. Lane C contained extract from *E. coli* containing the ANG/OHBD/Cys7Cys236 gene in the pET-11d expression plasmid.

Figure 9: SDS-PAGE analysis of the purified ANG/CM16/Cys7Cys236 gene product by Coomassie blue staining. Lane A contains standard protein molecular weight markers. Lanes B to E show ethanol-soluble extracts of the crude *E. coli* lysates. Lane B contained extract from cells containing the control plasmid, pET-11d. Lanes C, D and E contained ethanol soluble extracts of cells harbouring the pET-11d vector containing the ANG/CM16/Cys7Cys236 gene, prepared from cells 2, 4 and 6 hours, respectively, after induction of protein synthesis using IPTG.

Figure 10: Expression of the ANG/PIN-A/Cys7Cys236 gene. SDS-PAGE gel stained with Coomassie blue. Lane A contains standard protein molecular weight markers. Lane B contained extract of cells harbouring the control pET-11d plasmid, lanes C and D contained extracts of cells harbouring the pET-11d vector containing the ANG/PIN-A/Cys7Cys236 gene. Lane C contains extract 2 hours after IPTG induction, lane D contains extract prepared 6 hours following IPTG induction.

Figure 11: Expression of the ANG/PIN-A/Cys7Cys236 gene. SDS-PAGE analysis followed by Western blotting with anti-puroindoline A antibodies. Lanes A and B contained extract of cells harbouring pET-11d containing the ANG/PIN-A/Cys7Cys236 gene, 2 hours and 6 hours after induction respectively. Lane C contains a Western blot of cells harbouring a plasmid containing the puroindoline A gene alone (not inserted into the ANG Δ Cys7Cys236 gene). Lane D contained extract of cells harbouring the control pET-11d vector.

Figure 12: Expression of the ANG/PIN-A/Cys7Cys236 gene. SDS-PAGE analysis followed by Western blotting with anti-hordein antibodies. Lane A contains molecular weight markers. Lane B contained extract of cells harbouring the control pET-11d vector. Lane C contained extract of cells harbouring a plasmid which contains the puroindoline A gene alone. Lane D contained extract of cells harbouring pET-11d vector containing the ANG/PIN-A/Cys7Cys236 gene.

Figure 13: Plasmids for the transformation of wheat with constructs containing novel protein genes. The vectors are based on the plasmid pTLZHMWcas which has been modified by insertion of genes into the KpnI and BamHI site region. The vectors are: pTLZ-ANGCys7Cys13, pTLZ-ANGCys236 and pTLZ_ANG/OHBD/Cys7Cys236.

Figure 14: Expression and β -cyclodextrin affinity purification of ANG/SBD/Cys7Cys236. Native- and SDS-PAGE gels of column fractions from a 5 ml β -cyclodextrin-sepharose affinity column loaded with extracts from IPTG-induced cultures of strain AD494(DE3) bearing either pET11d control plasmid or the pET-SBD plasmid for the expression of ANG/SBD/Cys7Cys236. Panel A = SDS-PAGE of fractions from AD494(DE3)/pET-SBD. Panel B = native PAGE of fractions from AD494(DE3)/pET-SBD. Panel C = SDS-PAGE of fractions from AD494(DE3)/pET11d. Panel D = native PAGE of fractions from AD494(DE3)/pET11d. Lane M = molecular mass markers with masses indicated in kDa. Lane 1 = the unbound flow-through from column loading. Lanes 2-5 = four 5 ml washes with column buffer (50 mM Tris-Cl pH 7.5, 100 mM NaCl, 40 mM dithiothreitol, 1 mM EDTA). Lanes 6-10 = five 5 ml elutions with column buffer containing 15 mM β -cyclodextrin.

Figure 15: Schematic of the method of producing a modified seed storage protein containing a binding domain for a macromolecule. In this example, the C-hordein gene is altered to produce an analogue glutenin (ANG) protein containing cysteine residues in the N- and/or C-terminal domains. This protein is then further modified by replacement of part of its repetitive domain with a binding domain from another protein. The addition of cysteine residues allows for the incorporation of the protein into, for example, the gluten macropolymer in wheat flour. The addition of a specific binding domain provides for the non-covalent linkage of the gluten network to other macromolecules (e.g. starch, lipids). Such a protein may be expressed in recombinant bacteria, yeast, or other heterologous host for use as an ingredient or additive, or it may be produced in the seed of a transgenic plant, for example wheat.

Figure 16: Schematic of the scheme for identifying transgenes in transformed wheat plants by polymerase chain reaction (PCR). A primer pair straddling the interface between the gene and its promoter (from the gene for the high molecular weight glutenin Bx17) ensures that no false positives are detected arising from the high homology between C hordein and gliadin genes.

Figure 17: Agarose gel showing PCR-based identification of a transgenic wheat plant containing a gene for a modified seed storage protein with incorporated ligand-binding domain. In this example, the gene for ANG/OHBD/Cys7Cys236 was detected in the plasmid used for transforming the wheat plant (lane 1) and in a putative transformant wheat plant (lane 2), but is not detected in a non-transformed control wheat plant (lane 3).

Figure 18: Starch-binding activity of ANG/SBD/Cys7Cys236 protein assayed by native-PAGE. Protein from each of fraction 6 and 7 from the β -cyclodextrin affinity column of the urea extract of strain AD494(DE3)/pET-SBD was loaded onto native gels containing starch (panels B and D) and/or β -cyclodextrin (panels C and D) or neither starch nor β -cyclodextrin (panel A). The reduction in electrophoretic mobility in the presence of starch alone (panel B) is indicative of the specific starch-binding activity of each protein.

Figure 19: Demonstration of the oxidative polymerisation of a modified seed-storage protein containing a macromolecule-binding domain. In this example, ANG/SBD/Cys7Cys236 was oxidised to form disulphide-linked polymeric species (indicated by arrows in the scanned image of the

SDS-PAGE gel; lanes 1, 2) in the presence or absence of added β -cyclodextrin. The polymeric species were capable of being subsequently reduced by the disulphide-specific reductant dithiothreitol (DTT; lanes 3, 4), showing that the polymerisation was due to the presence of introduced

cysteine residues at the N- and C-termini of the ANG portion of the molecule.

M = molecular weight markers with masses indicated (kDa);

Lane 1 = ANG/SBD/Cys7Cys236 oxidised in the presence of β -cyclodextrin;

Lane 2 = ANG/SBD/Cys7Cys236 oxidised in the absence of β -cyclodextrin;

Lane 3 = ANG/SBD/Cys7Cys236 oxidised in the presence of β -cyclodextrin

and then reduced with DTT;

Lane 4 = ANG/SBD/Cys7Cys236 oxidised in the absence of β -cyclodextrin and then reduced with DTT;

Lane 5 = reduced ANG/SBD/Cys7Cys236 in the presence of β -cyclodextrin;

Lane 6 = reduced ANG/SBD/Cys7Cys236 in the absence of β -cyclodextrin.

Modes for Carrying Out the Invention

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli strain DH5 α was used as cloning host strain and *E. coli* strain AD494(DE3) (Novagen) was used as the expression host in this work.

pGEM-T (Promega) was used as cloning vector of PCR products.

pJKKm(-) (Kirschman and Cramer, 1988) served as cloning vehicle for fusion protein genes. New, assembled genes for expression were sub-cloned into plasmid pET-11d (Novagen).

Cloning of ANG Δ Cys7Cys236

Restriction endonucleases and DNA modifying enzymes were from New England Biolabs and Promega Corp. Other chemicals and reagents were of analytical reagent grade.

Oligonucleotide primers were synthesised on an Applied Biosystem 394 DNA/RNA Synthesiser using standard phosphoramidite chemistry and were deprotected by heating in ammonium hydroxide solution. Primers were lyophilised and dissolved in TE buffer.

To amplify up a 477 bp long fragment (approximately 2/3 of the whole gene) of C hordein (Accession X60037) from the genomic clone (Lambdahor1-17) of barley by polymerase chain reaction two specially designed primers were used.

oligonucleotide 1 (SEQ ID NO: 1):

Met Arg Cys
5' GTC ATG AGG CAA CTA AAC CCT TGC AGC CAA GAG TTG CAA TC
BspH I 3'

5 oligonucleotide 2 (SEQ ID NO: 2):

*** Val Cys Gln Gln Pro
5' GGA TCC CTA GAC CAT ACT CCA TAT GCA TGA AGC TTG TTG GGG
BamH I Nde I HindIII
GAC TGG TTG 3'

10 The reaction was performed in an FTS-4000 Thermal Sequencer (Corbett Research, Australia), with 1 cycle of 3 min at 94°C, 20 s at 55°C, 1 min at 72°C; 36 cycles of 30 s at 94°C, 1 min 30 s at 72°C. The reaction was carried out in 50 µl, containing 45 µl of Supermix (BRL Life Technology) and 1 ng of template DNA and 50 pmol of each oligonucleotides in 5 µl. The
15 DNA was purified following the QIAquick protocol (QIAGEN) and cloned, using the pGEM-T Vector System I (Promega) as was recommended by the manufacturer. White transformant colonies selected for growth in LB medium supplemented with ampicillin (100 mg/l) were screened for insert-bearing plasmid DNA by PCR. Plasmid DNA was purified from positive
20 clones using Jetstar miniprep columns (Genomed) and the insert was sequenced in both direction, using the Prism dye terminator cycle sequencing protocol (Perkin-Elmer). One plasmid isolate, containing the ANG gene, was designated as pGEM-ANG.

25 This plasmid contained two NdeI restriction sites, one within the gene and one within the pGEM-T multiple cloning site (MCS). To make it unique, the ANG gene was subcloned into pJKKm. Because pJKKm has a HindIII site in MCS, it had to be deleted before subcloning.

One ug of the plasmid was digested with 5 units of HindIII enzyme in 20 µl. Mung Bean Nuclease was used to remove the 5' overhang. Two µl of
30 10X ZnSO₄ solution and 0.02 units of enzyme was added to the reaction mix and incubated at 30°C. Reaction mix was extracted once with phenol/chloroform after one hour incubation. DNA was recovered with ethanol precipitation and resuspended in 50 µl. An aliquot of 10 µl of the
35 DNA solution was used for ligation. Ligation was carried out in 15 µl solution at 4°C overnight using T4 DNA ligase. The ligation mixture was used to transform competent cells of *E. coli* strain DH5α by electroporation.

Colonies were grown in LB medium supplemented with kanamycin (50 mg/l). Plasmid was purified from three colonies as mentioned above. DNA samples were tested by performing HindIII enzyme digestions. One clone not containing a HindIII site was chosen and designated as pJKK-H.

pJKK-H was cut with SphI and BamHI to subclone pGEM-ANG SphI-BamHI fragment into the plasmid. Both digested DNA were purified on QIAquick columns as described earlier, and ligated in 10 µl solution overnight at 14°C using T4 DNA ligase at a molar ratio of 50:1. Transformed *E. coli* competent cells was spread onto LB medium with kanamycin.

Colonies were tested for insert-bearing plasmid DNA by PCR and 3 positive clones were sequenced as above. One clone was designated as pJANGΔCys7Cys236 and used for further cloning work to assemble genes for fusion proteins. Nucleotide and amino acid sequences of this cloning vehicle are shown in Figure 1.

Design and cloning of oleosin hydrophobic binding domain

The sequence of oleosin hydrophobic binding domain (OHBD) was designed to include the consensus sequence of three (maize, rice and barley) oleosin proteins. The sequence is almost identical to the sequence published for barley oleosin isoform-2 (Aalen 1995, Accession Number X82678). Four primers were designed to encode the protein (Figure 2). The gene was constructed by a modification of the technique of overlap extension, where the two long partially overlapping oligonucleotides were further extended and amplified by shorter external primers, encoding restriction enzyme cleavage sites to clone into pJANGΔCys7Cys236. PCR amplification was performed as above, using the same cycle program and Supermix solution. Concentration of the long oligonucleotides were 0.1 nM, while 2 µM of the short primers in the PCR reaction mix. The PCR product was purified on QIAquick column and cloned into pGEM-T plasmid using Promega kit, as described above. Three positive clones were used for plasmid preparation and sequencing to confirm the nucleotide sequence. One clone was used for further work and called pGEM-OHBD.

Cloning of starch binding domain

The DNA corresponding to the starch binding domain (SBD) of Glucoamylase 1 (1,4-α-D-glucan glucohydrolase) of *Aspergillus niger* (Accession number: X00548) was amplified by PCR from purified genomic

DNA. Primers were designed to allow us to add tails at both ends of the DNA fragment for cloning into pJANGΔCys7Cys236 vector.

Sequence of the PCR primers are:

oligonucleotide 7 (SEQ ID NO: 3):

5 Gln Ala Cys Thr
5' CAA GCT TGT ACC ACT CCC ACC GCC 3'
 Hind III

oligonucleotide 8 (SEQ ID NO: 4):

 Ile Cys Arg
10 5' CCA TAT GCA CCG CCA GGT GTC AGT CAC 3'
 Nde I

Amplification, cloning into pGEM-T and sequencing was done as described above. One clone bearing the gene fragment was designated as pGEM-SBD.

15 **Cloning of CM16 and CM17 genes for fusion**

Both genes were amplified from purified wheat (*Triticum aestivum*) genomic DNA by PCR.

nucleotide 9 (SEQ ID NO: 5):

5' GTC GGC AAT GAA GAT TGC ACC 3'

20 nucleotide 10 (SEQ ID NO: 6):

5' TCC AAC TGC GTT CTC CTC TTG GCC 3'

nucleotide 11 (SEQ ID NO: 7):

5' GGA TCC CTA GCT CCA CTG AGA CTC 3'

25 For CM16 gene (accession number X55455) oligonucleotide 10 and 11, while for CM17 gene (accession number X59791) 9 and 11 pairs were used. Clones were called pGEM-CM16 and pGEM-CM17, respectively. For sub-cloning into pJANGΔCys7Cys236 vector, the genes were PCR amplified again, using pGEM clones as template, purified on QIAquick column and digested with the appropriate enzymes. Primers used in this amplification were:

30 nucleotide 12 (SEQ ID NO: 8):

 Gln Ala Leu Gly
5' TGC GCT CAA GCT TTA GGC AAT GAA GAT TGC ACC 3'
 Hind III

nucleotide 13 (SEQ ID NO: 9):

Ile Cys Ser

5' CAT ACT CCA TAT GCA GCT CCA CTG AGA CTC 3'

Nde I

5 Cloning puroindoline A gene for fusion

Lambda genomic clone for puroindoline A (PIN-A), kindly provided by Sadequer Rahman, was used as template to amplify the gene (accession number X69913) by PCR. Primers were designed as follows;

oligonucleotide 14 (SEQ ID NO: 10):

10 Gln Ala Tyr

5' CAA GCT TAC GAT GTT GCT GGC GGG 3'

Hind III

oligonucleotide 15 (SEQ ID NO: 11):

5' CCA TAT GCA CCA GTA ATA GCC AAT AGT GC 3'

15 Nde I

PCR product was purified, ligated into pGEM-T and sequenced as described above. One clone was used and designated as pGEM-PIN-A.

Fusion of genes or gene fragments with ANG molecule

pJANGΔCys7Cys236 vector was cut with NdeI and HindIII restriction enzymes as all the other pGEM clones and purified on QIAquick columns. Ligation was performed at 14°C overnight in 10 µl of solution containing T4 DNA ligase and insert:vector at about 20:1 molar ratio. One µl of ligation mix was used for transformation of *E. coli* competent cells and spread onto LB plate with kanamycin. Three fusion gene containing colonies were picked up from each transformation for plasmid preparation and sequencing. The clones were called, for example, pJANG/OHBD/Cys7Cys236 in case of the oleosin binding domain containing ANG molecule.

The fused genes were subcloned into pET-11d expression vector between NcoI and BamHI sites. These clones were called, for example, pET-ANG/OHBD/Cys7Cys236.

Sequence of PCR primers for detection of genes for ANG/domain/Cys7Cys236 in recombinant wheat

The primers used for the detection of genes for
ANG/domain/Cys7Cys236 in recombinant wheat are set out below.

5

Primer	Length	Sequence	Designation
Bx17_3'	23	CAACCATGTCCTGAACCTTCACC	SEQ ID NO:12
RGS2024	18	TGGCTGTTGAGGTTGCAC	SEQ ID NO:13

Expression of fusion proteins

For protein expression one of the pET clones were transformed into *E. coli* strain AD494(DE3) one day before expression work started.

10

Small scale expression was carried out in 5 ml 2YT medium, supplemented with ampicillin (100 mg/l) using one transformant colony. After about 5 hours from inoculation (OD₆₀₀=0.4), expression of the protein was induced by addition of 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Both induced and uninduced cultures were further incubated for 4

15

hours at 37°C. Expression was monitored by SDS-PAGE according to Laemmli (1970).

Large scale expression was performed in a shaking flask. One litre 2YT medium was inoculated with 1 ml of overnight culture and induced to express protein by addition of 0.4 mM IPTG at a cell density of ~0.6 Ab. The culture was incubated with shaking overnight and cells were harvested by centrifugation.

20

Detection of proteins

PAGE gels were stained overnight with 0.025% Coomassie Blue R-250 in 10% TCA. Excess stain was washed away by water-ethanol-acetic acid (8:1:1) solution.

25

Immunological detection of the PIN-A containing fusion protein was carried out using the method of Ciaffi et al., (1999). The antibody was raised against puroindoline crude extract, kindly provided by Sadequr Rahman.

Other chimeric proteins were detected in immunoblots using an antibody raised against Hordein, kindly provided by John Skerrett.

30

Purification of the fusion proteins

Expressed ANG/CM16/Cys7Cys236, ANG/CM17/Cys7Cys236 and ANG/SBD/Cys7Cys236 proteins were purified following the method published elsewhere (Tamas et al., 1998), except for the precipitation step. In this work 2 volumes of 1.5 M NaCl were mixed with the 70% ethanol extract, rather than 4 volumes of acetone.

Detection of the gene encoding modified seed storage proteins in transgenic wheat plants

The presence of the gene for modified proteins in transgenic wheat plants was determined by polymerase chain reaction (PCR). Reactions were carried out in 11.6 µl volume containing 9 µl PCR Supermix (GibcoBRL), 50 ng template DNA (extracted from wheat leaf tissue using standard protocols), 172 nmol of each of primers Bx17_3' and RGS2024, and 0.6 µl of 25 mM MgCl₂. The PCR conditions were 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min; 1 cycle of 72°C for 4 min, 25°C for 1 min. The results for one plant containing the gene for ANG/OHBD/Cys7Cys236 are shown in Figure 17. The PCR product of approximately 600 base pairs is indicative of the presence of the gene. A 600 bp PCR product is not obtained in PCR reactions from negative control plants.

RESULTS

Design, construction and cloning of pJANGΔCys7Cys236

The gene selected to construct this vector for fusion protein coded for C hordein. This molecule is a storage protein from barley endosperm and characterised by an absence of cysteine residues. The barley genomic clone encodes a molecule of 261 residues, including a 20 residue signal peptide. The gene for mature protein (molecular mass 28 kDa) has 723 nucleotides, including a 669 bp long fragment for a central repetitive domain. The oligonucleotides for PCR were designed to reduce the size of the central part and substitute one residue by cysteine in both unique terminal domains. Oligonucleotide 1 binds to the 5' end of the gene and has an additional sequence at the 5' end to incorporate an initiation ATG codon for methionine and a restriction site for BspHI. Oligonucleotide 1 has a T at position 22 to replace an A to change the codon of serine to cysteine at position 7. The 3' end of oligonucleotide 2 is complimentary to a sequence in the repetitive domain between 430 and 448 nucleotides. This is a rather unique sequence

within the strongly repetitive region and also codes for an end of a repeat motif. The amplified gene contains only a 411 bp (137 amino acids) long fragment for central repetitive domain. This oligonucleotide also contains the whole sequence of the C-terminal unique region (6 amino acids) of C hordein and a restriction site for BamHI, immediately after the stop codon. To change the threonine residue at the position of 236 of the full size molecule (six residues from the C-terminal end) the oligonucleotide has a C at position 26 and an A at position 27 to substitute a G and a T, respectively. There is another base pair change in this primer at position 22 to replace C with A, which is a "wobble" base of the isoleucine codon. This substitution allowed the creation of restriction site for NdeI enzyme. Oligonucleotide 2 has also got six nucleotides which are not part of the C hordein gene, coding for alanine and serine. These extra nucleotides were added to create one unique restriction site, close to NdeI, within the ANG gene. The two restriction sites are separated with 4 bp to give easy cleavage for both enzymes. The insertion of a gene for another molecule or fragment of a molecule between HindIII and NdeI enabled the present inventors to create fusion proteins with new, designed characteristics.

The gene for ANG Δ Cys7Cys236 molecule is 474 bp long and codes for a protein with a molecular mass of 18.5 kDa (Figure 3).

PCR amplified DNA was cloned, with two steps, into a plasmid, called pJKKm with a modified MCS. Having deleted HindIII restriction site from the original plasmid, the cloning vehicle pJANG Δ Cys7Cys236 has unique HindIII and NdeI cleavage sites within ANG gene for gene insertion. Size of the vector is 3873 bp and provides kanamycin resistance to host *E. coli* cells. The gene is sitting in the vector with 5' ends close to the SP6 RNA Polymerase transcription initiation site.

Subcloning engineered genes into pET-11d did not require agarose gel purification of the fragments, because of the difference in resistance genes within plasmids.

Expression of ANG Δ Cys7Cys236 protein

To check and compare characteristics of this short molecule to hordeinCys7Cys236, it has been expressed in both small and large quantities. Comparison of the SDS-PAGE patterns of the total cell proteins before and 3 hours after induction with IPTG showed a new band in the induced sample. ANG molecule was readily extracted from lysed cells with 70% (v/v) ethanol

and precipitated by the addition of 2 volume of 1.5 M NaCl solution. The resulting preparation, in the presence of reducing agent (0.1 M DTT), had the same mobility as the extra band in *E. coli* lysate. Apparent molecular mass of the protein was about 21 kDa, having a slightly lower mobility in the gel.

5 This characteristic is not unusual for storage proteins. In the absence of DTT, the ethanol extracted sample gave a ladder of bands indicating that the protein is able to form long chains through disulfide bonds.

The ability of the ANG molecule to incorporate into the gluten matrix of the dough was confirmed by a series of mixing experiments carried out using small scale testers.

10 Results of SDS-PAGE and mixing experiments showed clearly that ANG Δ Cys7Cys236 protein had the same or similar properties as the 2 cysteine residue containing C hordein protein.

Design, construction and cloning of synthetic gene for oleosin hydrophobic binding domain (OHBD)

15 Amino acid sequence was designed according to a comparison of four molecules (one maize, two barleys and one rice), using Genetic Computer Group (GCG) program, called "pileup". OHBD gene fragment, for this work, contain the entire region of the lipophilic stretch of oleosin (Figure 2), very similar to barley gene (accession number: X82677). Codon usage was designed to avoid long stretch of Gs and Cs, which could have led to mis-annealing and sequencing problems. The 5' flanking region of the gene fragment consisted of a tripeptide sequence, of the amphipathic N terminus, while the 3' end another tripeptide of the C terminus of oleosin. The central anti-parallel beta stranded domain had 71 residues. The turn of the anti-parallel consists of 13 residues and is the most conserved region. The two anti-parallel strands are highly symmetrical in the pairing of residues of similar hydrophobicity on the opposite strands. These characteristics are very similar to those reported for maize oleosin protein (Huang 1996).

25 30 The synthetic gene fragment, flanked with two appropriate restriction sites for subcloning, was cloned into pGEM-T vector. One clone, containing the correct sequence of a fragment of 243 bp nucleotides, was designated as pGEM-OHBD. Size of OHBD fragment was 77 amino acids and had a molecular mass of 7 kDa.

Cloning of starch binding domain (SBD)

Glucoamylase 1 from *Aspergillus niger* comprises two domains, one being a catalytic domain (1-470 residues) and the other (509-616 residues) being responsible for binding granular starch (Le Gal-Coeffet et al., 1995).

5 DNA corresponding to the SBD was amplified by PCR method from *Aspergillus niger* purified genomic DNA, cloned into pGEM-T vector, sequenced and designated as pGEM-SBD. This clone had a 337 bp long fragment (Figure 4) with two restriction sites for insertion into pJANGΔCys7Cys236 vector and an extra alanine before the very first cysteine of SBD. This residue derived from HindIII restriction site. The binding domain had 108 amino acids, including two cysteines, with a molecular mass of 11.9 kDa.

Cloning of CM16 and CM17 gene for fusion

Both CM16 and CM17 (CM refers to Chloroform/Methanol soluble) proteins, reported as members of the α -amylase/trypsin inhibitor family and also reported that specific lipids are tightly bound to the fraction, were purified from wheat (Kobrehel and Sauvaire, 1990). These two molecules are very similar on the amino acid level, but there are a few differences in the distribution of charged residues (Figure 5).

20 To clone these two genes, three primers were used to amplify them in two, separate PCR reactions from wheat genomic DNA. One primer (oligonucleotide 11) hybridised to the 3' end of the genes, while to distinguish between the two genes, two specific primers were designed for the 5' ends. pGEM-CM17 clone carried the gene for only the mature protein. 25 However, pGEM-CM16 clone had a few extra base pairs from the signal peptide region. Both clones had a DNA fragment encoding mature chloroform/methanol soluble proteins with 10 cysteine residues and a molecular mass of 13.4 kDa.

To subclone these genes into ANGΔCys7Cys236 carrying vector, two 30 restriction sites were added to one of each ends by PCR, using the same primer pairs for both pGEM clones, as templates. Primer corresponding to the N-terminus of the mature proteins contained nucleotides for HindIII restriction enzyme. It also had an extra alanine residue, and a mutation in the first codon (TTA for leucine), substituting valine (GTC) in CM17 and 35 isoleucine (ATC) in CM16 molecule, because of the sequence requirement for

HindIII enzyme. The fragment cloned into ANG cloning vehicle had 360 nucleotides.

Cloning puroindoline A (PIN-A) gene for fusion

The clone for PIN-A protein, which is capable of binding tightly to both wheat phospholipids and glycolipids (Dubreil et al., 1997), was kindly provided by S. Rahman, derived from a genomic library.

PCR amplified DNA encoded for a 121 amino acid long fragment of puroindoline A, 5 residues shorter in the N terminal region than the mature protein. This shorter protein had 10 cysteine residues and a molecular mass of 14.3 kDa (Figure 6). For insertion into ANG molecule, the pGEM-PIN-A clone also carried two restriction sites (HindIII and NdeI) and an extra alanine, because of HindIII site requirement.

Expression of ANG/OHBD/Cys7Cys236 protein

Having inserted OHBD gene fragment into pJANGΔCys7Cys236 cloning vehicle, the entire gene was subcloned into expression vector pET-11d. Protein was expressed in *E. coli*, using AD494(DE3) strain. This genetically engineered new gene (717 bp long) coded for a protein with a molecular mass of 25.5 kDa.

Cells were harvested by centrifugation after overnight expression (5 ml culture) and resuspended in 100 μ l gel loading buffer (0.125 M TRIS/HCl pH 6.8, 4% SDS, 10% (v/v) glycerol), containing 0.1 M DTT. Proteins were analysed by SDS-PAGE and stained with Coomassie blue (Figure 7). Lane A contains standard molecular weight markers, lane B shows a control in which the host *E. coli* cell contains the pET-11d vector, and lane C shows the expression of the recombinant ANG/OHBD/Cys7Cys236 protein (migrating slightly further than the 30 kDa marker) from pET-11d containing the ANG/OHBD/Cys7Cys236 gene. The apparent molecular mass of the newly synthesised protein (~29 kDa) was larger than that calculated on the basis of the nucleotide sequence of DNA. A similar characteristic has been observed previously for different prolamines of cereals. This fusion protein consisted of part of a prolamine and a part of an oleosin molecule. C hordein had been considered as a rod shaped molecule, while oleosin hydrophobic binding domain has anti-parallel strands, which would penetrate into the oil body and thus anchor the protein stably. It means that none of the two fragments has globular structure as compared with the molecular weight marker molecules. Discrepancy in apparent molecular mass on SDS-PAGE was

probably due to these two structural phenomena and it was manifested in lower mobility. Figure 8 shows a Western blot of an SDS-PAGE gel (run in the absence of reducing agents such as dithiothreitol) using antibodies to the C-hordein protein. Lane A contained standard molecular weight markers, lane B contained extract of an *E. coli* cell expressing a modified C-hordein gene containing a single cysteine in the N-terminal region, and lane C contained extract from *E. coli* containing the ANG/OHBD/Cys7Cys236 gene in the pET-11d expression plasmid. The strong antibody cross reaction to hordein antibodies at 29 kDa confirms that the 29 kDa protein is the product of the ANG/OHBD/Cys7Cys236 gene.

Expression and analysis of ANG/PIN-A/Cys7Cys236 protein

A DNA fragment comprised of ANG Δ Cys7Cys236, and Puroindoline-A genes with a length of 840 nucleotides from kanamycin resistant plasmid (see above) was subcloned into pET-11d expression vector. One transformant colony was picked up and transferred into 5 ml of 2YT medium. Protein expression was induced by IPTG and after 2 and 6 hours cells were harvested by centrifugation and resuspended in 200 μ l of gel loading buffer. SDS-PAGE analysis of protein content of bacteria, before and after induction, is shown on Figure 10. Lane A contained standard protein molecular mass markers. Lane B contained extract of cells harbouring the control pET-11d plasmid, lanes C and D contained extracts of cells harbouring the pET-11d vector containing the ANG/PIN-A/Cys7Cys236 gene. Lane C contained extract 2 hours after IPTG induction, lane D contained extract prepared 6 hours following IPTG induction. An extra protein band appeared on gel with an apparent molecular mass of 35 kDa, after induction (lane D). This expressed protein positively reacted with puroindoline A antibody (Figure 11). Lanes A and B contained extract of cells harbouring pET-11d containing the ANG/PIN-A/Cys7Cys236 gene, 2 hours and 6 hours after induction respectively. Lane C contained a Western blot of cells harbouring a plasmid containing the puroindoline A gene alone (not inserted into the ANG Δ Cys7Cys236 gene), and lane D contained extract of cells harbouring the control pET-11d vector. All lanes were reacted with anti- puroindoline A antibody. Figure 12 shows a Western blot of an SDS-PAGE gel reacted with anti-hordein antibodies. Lane A contained molecular mass markers, lane B contained extract of cells harbouring the control pET-11d vector, lane C contained extract of cells harbouring a plasmid which contains the

puroindoline A gene alone, and lane D contained extract of cells harbouring pET-11d vector containing the ANG/PIN-A/Cys7Cys236 gene. The expressed protein is clearly seen in lane D.

Analogue glutenin-puroindoline A fusion protein had a slightly lower mobility on SDS-PAGE as it was calculated (32.8 kDa) on the basis of amino acid sequence. This small difference was probably due to the rod shape of the ANG part of the molecule. Expressed puroindoline A protein, however, had the same mobility as it was calculated.

Expression of modified proteins following wheat transformation

Microprojectile bombardment is currently the most widely applied technique used to transfer genes into wheat. The transfer of genes into wheat and their expression is carried out using specific DNA constructs containing a selectable marker gene and the gene-of-interest respectively. The selectable marker gene used was contained on the plasmid pEmuKON (Chamberlain et al., 1994). Constructs for the gene-of-interest constructs are shown in Figure 13. Wheat transformation was carried out using paromomycin selection according to the procedures outlined in Witrzens et al., 1998).

Expression, purification and analysis of ANG/SBD/Cys7Cys236 protein

PET11d and the derivative of that plasmid containing the gene for ANG/ SBD/Cys7Cys236 protein (pET-SBD) were separately transformed into strain AD494(DE3). One transformant colony of each was used to inoculate 5 ml cultures of LB media containing 200 mg/l ampicillin. After 4 hours shaking at 37°C these cultures were used to inoculate 600 ml of the same media in 2 l flasks, and these were incubated with shaking overnight (16 hours) at 37°C. IPTG was added to these cultures 6 hours after inoculation to induce expression of protein from the plasmids. Cells from each culture were harvested by centrifugation at 10,000 g for 15 min at 5°C.

One half of the cells from each culture were lysed by incubation for 30 min at room temperature in 35 ml of 50 mM Tris-Cl buffer pH 7.5 containing 8 M urea, 5 mM EDTA and 1 mM DTT. Insoluble material was removed by centrifugation at 2×10^5 g for 1 hr at 15°C. The supernatants were then extensively dialysed against the extraction buffer minus urea (50 mM Tris-Cl pH 7.5, 5 mM EDTA, 1 mM DTT) at 5°C. The cloudy precipitates were removed by centrifugation at 2×10^5 g for 1 hr at 5°C and the supernatants (35 mL) stored at -20°C.

β -Cyclodextrin-binding proteins were purified from 20 ml of each of the two dialysed and clarified bacterial extracts by passage through a 5 ml β -cyclodextrin-sepharose column equilibrated with 50 mM Tris-Cl pH 7.5, 100 mM NaCl, 40 mM dithiothreitol, 1 mM EDTA (column buffer). After loading, the column was washed with 20 ml column buffer and bound proteins eluted with 25 ml column buffer containing 15 mM β -cyclodextrin. Five ml fractions were collected during loading, washing and elution of the column. Fractions were dialysed extensively against water, lyophilised and resuspended in 0.1 M Tris-Cl pH 6.8 at ca. 1 μ l per ml equivalent of the original bacterial cell culture.

Native- and SDS-PAGE gels of the final load fraction and the wash and elution fractions are shown in Figure 14. Each lane contained protein from the equivalent of 7 ml of the bacterial culture. Two major β -cyclodextrin-binding proteins were seen in the elution fractions (see lanes 6 and 7) from the strain bearing plasmid pET-SBD. These are estimated to be 30 kDa and >60 kDa from the SDS-PAGE gel. A 30 kDa protein was also eluted from the column of the extract from the control strain bearing plasmid pET11d (panel C, lane 6), but it is not produced to the same level as in the former strain.

Expression, purification and analysis of ANG/CM16/Cys7Cys236 protein

The gene for this fusion protein (834 bp long) was subcloned, from kanamycin resistant, pJKKm originated plasmid, into the ampicillin resistant pET-11d expression vector, as above. Bacteria was grown at 37°C until OD600 reached 0.6 units, then expression was induced by adding IPTG. Cells were harvested two, four or six hours after induction and samples were prepared as in case of SBD chimeric protein.

Result of the SDS-PAGE analysis is shown in Figure 9 (the gel is stained with Coomassie protein stain). Lane A contains standard protein molecular weight markers. Lanes B to E show ethanol-soluble extracts of the crude *E. coli* lysates. Lane B contained extract from cells containing the control plasmid, pET-11d. Lanes C, D and E contained ethanol soluble extracts of cells harbouring the pET-11d vector containing the ANG/CM16/Cys7Cys236 gene, prepared from cells 2, 4 and 6 hours respectively after induction of protein synthesis using IPTG. The ethanol-soluble fraction gave only one band on Coomassie stained gel representing a protein with an apparent molecular mass of 35 kDa. This was also higher

than the calculated (31.9 kDa), and this discrepancy is also explained by the unusual shape of the protein.

Yield of protein expression in large scale, in shaking flask was 30 mg protein from 1 litre of medium.

5 **Demonstration of Starch Binding Activity**

The starch-binding activities of the major proteins in fractions 6 and 7 of the affinity purification column were demonstrated as follows. Fractions were loaded onto four native-PAGE gels containing glycerol (10% w/v), CHAPS (0.05% w/v) and various combinations of β -cyclodextrin (5 mM) and starch (corn amylopectin, 0.1% w/v), as indicated in Figure 18. The mobility of the protein bands in each gel was compared. It was found that the electrophoretic mobility of the major protein band in each fraction was significantly retarded in the presence of starch (Figure 18, panel B). The specificity of the starch binding activity of these protein bands was demonstrated by its competitive inhibition by β -cyclodextrin (panels C, D).

15 **Identification of the purified starch-binding proteins by tryptic-peptide mass fingerprinting and N-terminal protein sequencing**

The two major protein bands from the native gel of β -cyclodextrin affinity column fractions from strain AD494(DE3)/pET-SBD were subjected to in-gel tryptic digestion followed by matrix-assisted laser-desorption time-of-flight (MALDI-TOF) mass spectroscopy. The masses of the identified tryptic fragments were compared to the theoretical masses of tryptic peptides expected from ANG/SBD/Cys7Cys236, as well as being used to search the protein sequence databases for other candidate proteins giving matching tryptic peptides.

The 30 kDa protein of fraction 6 (Figure 14, panels A, B, lane 6) was identified as the periplasmic maltose-binding protein of *E. coli* (product of the *malE* gene), based on 17 peptide matches covering 57% of the protein sequence.

The major protein band of fraction 7 (Figure 14, panels A, B, lane 7) was identified as ANG/SBD/Cys7Cys236 on the basis of 4 matching peptides covering 23.11% of the sequence (Table 1). Further proof of the identity of this protein was obtained from N-terminal protein sequencing. Ten cycles of sequencing yielded the sequence:

35 M R Q L N P C S Q E

which is as expected for ANG/SBD/Cys7Cys236. This provides convincing proof of the identity of the purified protein, and confirms that the functional data are properties of the modified seed storage protein, as designed.

5 Table 1. Mass spectral characterisation of ANG/SBD/Cys7Cys236

Peptide mass	Expected mass	Start	End	Sequence
1776.74	1777.28	224	238	IESDDSVESWESDPNR
2193.00	2193.85	221	238	FIRIESDDSVESWESDPNR
2652.26	2653.54	198	220	YTSSDPLWYVTVTLPAGESEYK
2257.03	2258.00	239	258	EYTVLPQAC*GTSTATVTDTR

C* = acrylamide adduct of cysteine

10 **Demonstration of the ability of ANG/SBD/Cys7Cys236 to form disulphide-bonded polymers**

The design of the analogue glutenin protein (ANG) included cysteine residues at both N- and C-termini to allow disulphide-mediated incorporation of the modified seed-storage protein into the gluten macropolymer. The present inventors have shown by *in vitro* polymerisation experiments that the ability of the ANG/domain/Cys7Cys236 proteins to incorporate into disulphide-bonded polymeric species is not impaired by the presence of the extra domain.

The ability to form polymers was demonstrated by copper-mediated oxidation of ANG/SBD/Cys7Cys236 protein in 50 mM Tris-Cl buffer pH 6.8. Three pairs of protein samples were employed, with one of each pair containing added β -cyclodextrin (0.5 mM) to analyse any effect of a ligand on the disulphide-bonding of the ANG. Two pairs were oxidised for 5 min at room temperature in the presence of 0.1 mM CuSO₄, before chelation of the metal by addition of EDTA (10 mM). One of these pairs was then reduced in the presence of 20 mM DTT. A further pair of samples had no copper added, but were reduced with DTT in the presence of EDTA. The samples were analysed by SDS-PAGE (Figure 19). There was no effect on oxidation due to the presence of the ligand β -cyclodextrin. The majority of the oxidised protein sample forms a high molecular weight band which just enters the separating gel (lanes 1, 2). That this change in mobility is due to the

formation of disulphide-bond polymeric forms is shown by the ability of the disulphide-specific reductant DTT (lanes 3, 4) to break the polymer back down into the higher-mobility form seen in the reduced sample (lanes 5, 6).

Detection of the gene encoding modified seed storage proteins in transgenic wheat plants.

The presence of the gene for modified proteins in transgenic wheat plants was determined by polymerase chain reaction (PCR). Reactions were carried out in 11.6 μ l volume containing 9 μ l PCR Supermix (GibcoBRL), 50 ng template DNA (extracted from wheat leaf tissue using standard protocols), 172 nmol of each of primers Bx17_3' and RGS2024, and 0.6 μ l of 25 mM $MgCl_2$. The PCR conditions were 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min; 1 cycle of 72°C for 4 min, 25°C for 1 min. The results for one plant containing the gene for ANG/OHBD/Cys7Cys236 are shown in Figure 17. The PCR product of approximately 600 base pairs is indicative of the presence of the gene. A 600 bp PCR product is not obtained in PCR reactions from negative control plants.

Demonstration of the ability of ANG/SBD/Cys7Cys236 to form disulphide-bonded polymers

The design of the analogue glutenin protein (ANG) included cysteine residues at both N- and C-termini to allow disulphide-mediated incorporation of the modified seed-storage protein into the gluten macropolymer. The present inventors have shown by *in vitro* polymerisation experiments that the ability of the ANG/domain/Cys7Cys236 proteins to incorporate into disulphide-bonded polymeric species is not impaired by the presence of the extra domain.

The ability to form polymers was demonstrated by copper-mediated oxidation of the protein in 50 mM Tris-Cl buffer pH 6.8. Three pairs of protein samples were employed, with one of each pair containing added β -cyclodextrin (0.5 mM) to analyse any effect of ligand-binding on the disulphide-bonding of the ANG moiety. Two pairs were oxidised for 5 min at room temperature in the presence of 0.1 mM $CuSO_4$, before chelation of the metal by addition of EDTA (10 mM). One of these pairs was then reduced in the presence of 20 mM DTT. A further pair of samples had no copper added, but were reduced with DTT in the presence of EDTA. The samples were analysed by SDS-PAGE (Figure19). There was no effect on oxidation due to

the presence of the ligand β -cyclodextrin. The majority of the oxidised protein sample forms a high molecular weight band which just enters the separating gel (lanes 1, 2). That this change in mobility is due to the formation of disulphide-bond polymeric forms is shown by the ability of the disulphide-specific reductant DTT (lanes 3, 4) to break the polymer back down into the higher-mobility form seen in the reduced samples (lanes 5, 6).

SUMMARY

The demonstration of the binding activity of a modified glutenin according to the present invention was provided in the example given above for what has been termed the ANG/SBD/Cys7Cys236 protein. The data provided supports the demonstration of the expression and purification of a novel protein (apparent molecular mass 60 kDa) from the *E. coli* strain bearing the expression plasmid (pET-SBD), in comparison to the lack of this protein in the same strain bearing the control plasmid (pET11d) (Figure 14). The purification involved the use of an affinity chromatography step in which the protein was purified on the basis of its ability to bind to a column matrix containing β -cyclodextrin. The protein was specifically eluted from this column using unbound β -cyclodextrin. This, therefore, represents the first example of how the present inventors demonstrate reduction to practice through the generation of a seed storage protein containing a functional ligand binding domain.

The demonstration of the binding of the major purified proteins to corn starch using native polyacrylamide gel electrophoresis, and the inhibition of this binding by β -cyclodextrin (PAGE) (Figure18) is a highly convincing demonstration of reduction to practice of the present invention.

The identification of this protein as ANG/SBD/Cys7Cys236 by peptide mass fingerprinting and N-terminal protein sequencing further demonstrates the functionality of modified seed-storage proteins containing a macromolecular binding domain. The anomolous apparent molecular weight of this protein in SDS-PAGE may be yet another example of the general phenomenon of modified C hordein proteins migrating with unexpected electrophoretic mobility, as the calculated mass of the protein is only 30 kDa. Alternatively, the protein may form a dimer, perhaps around a single bound β -cyclodextrin ligand, which is not dissociated by boiling in the presence of SDS and a reducing agent.

The ANG portion forms disulphide-bonded polymeric species was demonstrated by native-PAGE of the copper-oxidised affinity purified proteins. The fusion protein has been demonstrated to contain the novel starch binding activity. Importantly, it is believed that the modified protein has not lost the ability to polymerise through the cysteine residues at the N- and C-termini and therefore the novel protein can be incorporated into the gluten macropolymer in wheat flour.

Demonstration of the capacity of the modified seed-storage protein with macromolecule-binding domain (in this instance ANG/SBD/Cys7Cys236) to form disulphide-bonded polymeric species (Figure 19). This demonstrates the ability of the protein to be incorporated into the gluten macropolymer through disulphide bonds.

Table 1 demonstrates the identity between the molecular weights of the experimentally determined tryptic fragments of the major β -cyclodextrin-binding protein (starch-binding protein) purified from the ANG/SBD/Cys7Cys236 strain and the theoretical masses of the tryptic fragments of the ANG/SBD/Cys7Cys236 protein. The identification of the 30kDa protein as an endogenous bacterial maltose-binding protein is also provided.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Commonwealth Scientific and Industrial Research Organisation

(B) STREET: Limestone Avenue

(C) CITY: Campbell

(D) STATE: ACT

(E) COUNTRY: Australia

(F) POSTAL CODE (ZIP): 2601

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(B) STREET: Level 42 Grosvenor Place

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(D) STATE: NSW

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(F) POSTAL CODE (ZIP): 2000

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(C) CITY: Sydney

(D) STATE: NSW

(E) COUNTRY: Australia

(F) POSTAL CODE (ZIP): 2000

(ii) TITLE OF INVENTION: Modified Proteins

(iii) NUMBER OF SEQUENCES: 26

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GTCATGAGGC AACTAAACCC TTGCAGCCAA GAGTTGCAAT C

41

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 51 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGATCCCTAG ACCATACTCC ATATGCATGA AGCTTGTTGG GGGACTGGTT G

51

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CAAGCTTGTA CCACTCCCAC CGCC

24

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCATATGCAC CGCCAGGTGT CAGTCAC

27

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTCGGCAATG AAGATTGCAC C

21

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCCAAGTGGC TTCTCCTCTT GGCC

24

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGATCCCTAG CTCCACTGAG ACTC

24

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TGCGCTCAAG CTTTAGGCAA TGAAGATTGC ACC

33

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CATACTCCAT ATGCAGCTCC ACTGAGACTC 30

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CAAGCTTACG ATGTTGCTGG CGGG 24

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCATATGCAC CAGTAATAGC CAATAGTGC 29

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CAACCATGTC CTGAACCTTC ACC 23

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TGGCTGTTGA GGTTCAC

18

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 140 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATAGAATACA GCATGCTCCC GGCCGCCATG GCCGCGGGAT TGTCATGAGG CAACTAAACC 60
 CTTGCAGCGT CCCCCAACAA GCTTCATGCA TATGGAGTAT GGTCTAGGGA TCCGGGTACC 120
 GAGCTCGAAT TCGCCCTATA 140

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Met	Arg	Gln	Leu	Asn	Pro	Cys	Ser	Val	Pro	Gln	Gln	Ala	Ser	Cys	Ile
1				5					10					15	
Trp Ser Met Val															
20															

(2) INFORMATION FOR SEQ ID NO: 16:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 243 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```

CAAGCTTTGA AAGCCGCTAC TGCGACAGCA GCTGGATCAT TGCTTGTGCT ATCCGGACTA  60
ATACTAGCTG GCACAGTCAT AGCACTCACA GTGGCCACAC CAGTGCTAGT CATATTTAGC 120
CCAGTGCTAG TGCCAGCGGC CATAGCCCTA GCGCTAATGT CAGCAGGCTT TGTCACGTCA 180
GGCGGGGCTGG GCGTGGCTGC GCTGAGCTCC TTTAGTGTGT TAGCCAATAC TGCCTGCATA 240
TGG

```

243

(2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 81 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

```

Gln Ala Leu Lys Ala Ala Thr Ala Thr Ala Ala Gly Ser Leu Leu Val
 1              5              10              15
Leu Ser Gly Leu Ile Leu Ala Gly Thr Val Ile Ala Leu Thr Val Ala
          20              25              30
Thr Pro Val Leu Val Ile Phe Ser Pro Val Leu Val Pro Ala Ala Ile
          35              40              45
Ala Leu Ala Leu Met Ser Ala Gly Phe Val Thr Ser Gly Gly Leu Gly
          50              55              60
Val Ala Ala Leu Ser Ser Phe Ser Val Leu Ala Asn Thr Ala Cys Ile
65              70              75              80
Trp

```

(2) INFORMATION FOR SEQ ID NO: 18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 477 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

```

ATGAGGCAAC TAAACCCTTG CAGCCAAGAG TTGCAATCAC CACAACAATC ATATCTGCCG   60
CAGCCATATC CACAAAACCC ATATCTACCG CAAAACCAT TTCCAGTGCA GCAACCGTTT  120
CACACACCCC AACAATATTT CCCCTATCTA CCAGAGGAAT TGTTTCCCCA ATATCAAATA  180
CCAACCCCCC TACAACCACA ACAACCATTC CCCCAACAAC CACAACAACC TCTTCCTCGG  240
CCCCAACAAC CATTCCCCTG GCAACCACAA CAACCATTTT CCCAGCCCCA AGAACCAATT  300
CCCCAACAAC CATTCCCCTG GCAACCACAA CAACCATTTT CCCAGCCCCA AGAACCAATT  360
CAACAAATAA TTTTCCAGCA ACCCCAACAA TCATACCCTG TGCAACCTCA ACAGCCATTT  420
CCTCAACAAC CTCAACCAGT CCCCCAACA GCTTCATGCA TATGGAGTAT GGTCTAG    477

```

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 158 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

```

Met Arg Gln Leu Asn Pro Cys Ser Gln Glu Leu Gln Ser Pro Gln Gln
1           5           10           15
Ser Tyr Leu Gln Gln Pro Tyr Pro Gln Asn Pro Tyr Leu Pro Gln Lys
          20           25           30
Pro Phe Pro Val Gln Gln Pro Phe His Thr Pro Gln Gln Tyr Phe Pro
          35           40           45
Tyr Leu Pro Glu Glu Leu Phe Pro Gln Tyr Gln Ile Pro Thr Pro Leu
          50           55           60
Gln Pro Gln Gln Pro Phe Pro Gln Gln Pro Gln Gln Pro Leu Pro Arg
          65           70           75           80
Pro Gln Gln Pro Phe Pro Trp Gln Pro Gln Gln Pro Phe Pro Gln Pro
          85           90           95

```

Gln Glu Pro Ile Pro Gln Gln Pro Gln Gln Pro Phe Pro Gln Gln Pro
 100 105 110

Gln Gln Pro Phe Pro Gln Gln Pro Gln Gln Ile Ile Phe Gln Gln Pro
 115 120 125

Gln Gln Ser Tyr Pro Val Gln Pro Gln Gln Pro Phe Pro Gln Gln Pro
 130 135 140

Gln Pro Val Pro Gln Gln Ala Ser Cys Ile Trp Ser Met Val
 145 150 155

(2) INFORMATION FOR SEQ ID NO: 20:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 338 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AAGCTTCTAC CACTCCCACC GCCGTGGCTG TGA CTTTCGA TCTGACAGCT ACCACCACCT 60
 ACGGCGAGAA CATCTACCTG GTCGGATCGA TCTCTCAGCT GGGTGACTGG GAAACCAGCG 120
 ACGGCATAGC TCTGAGTGCT GACAAGTACA CTTCCAGCGA CCCGCTCTGG TATGTCACTG 180
 TGA CTTCTGCC GGCTGGTGAG TCGTTTGAGT ACAAGTTTAT CCGCATTGAG AGCGATGACT 240
 CCGTGGAGTG GGAGAGTGAT CCCAACCGAG AATACACCGT TCCTCAGGCG TCGGGAACGT 300
 CGACCGCGAC GGTGACTGAC ACCTGGCGGT GCATATGG 338

(2) INFORMATION FOR SEQ ID NO: 21:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 112 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Ala Ser Thr Thr Pro Thr Ala Val Ala Val Thr Phe Asp Leu Thr Ala
 1 5 10 15

Thr Thr Thr Tyr Gly Glu Asn Ile Tyr Leu Val Gly Ser Ile Ser Gln
 20 25 30

```

Leu Gly Asp Trp Glu Thr Ser Asp Gly Ile Ala Leu Ser Ala Asp Lys
    35                                40                                45

Tyr Thr Ser Ser Asp Pro Leu Trp Tyr Val Thr Val Thr Leu Pro Ala
    50                                55                                60

Gly Glu Ser Phe Glu Tyr Lys Phe Ile Arg Ile Glu Ser Asp Asp Ser
    65                                70                                75                                80

Val Glu Trp Glu Ser Asp Pro Asn Arg Glu Tyr Thr Val Pro Gln Ala
                                85                                90                                95

Cys Gly Thr Ser Thr Ala Thr Val Thr Asp Thr Trp Arg Cys Ile Trp
    100                                105                                110

```

(2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 371 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

```

AAGCTTTCGG CAATGAAGAT TGCACCCCAT GGATGAGTAC TCTGATCACT CCACTCCCAA 60
GCTGCCGTGA CTATGTGGAA CAACAAGCAT GTCGCATCGA AACGCCCGGG TCGCCGTACC 120
TCGCCAAGCA GCAGTGCTGT GGGGAGCTTG CAAACATTCC GCAGCAGTGC CGATGCCAGG 180
CGCTGCGCTA CTTTCATGGGG CCGAAGTCTC GTCCGGATCA GAGCGGCCTC ATGGAAGTCC 240
CCGGATGCCC TAGGGAGGTG CAGATGGACT TCGTGAGGAT ACTCGTCACG CCGGGGTACT 300
GCAACTTGAC GACCGTTCAC AACACTCCGT ACTGCCTCGC TATGGAGGAG TCTCAGTGGA 360
GCTGCATATG G

```

371

(2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 123 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

```

Ala Ile Gly Asn Glu Asp Cys Thr Pro Trp Met Ser Thr Leu Ile Thr
1           5           10
Pro Leu Pro Ser Cys Arg Asp Tyr Val Glu Gln Gln Ala Cys Arg Ile
          20           25           30
Glu Thr Pro Gly Ser Pro Tyr Leu Ala Lys Gln Gln Cys Cys Gly Glu
          35           40           45
Leu Ala Asn Ile Pro Gln Gln Cys Arg Cys Gln Ala Leu Arg Tyr Phe
          50           55           60
Met Gly Pro Lys Ser Arg Pro Asp Gln Ser Gly Leu Met Glu Leu Pro
65           70           75           80
Gly Cys Pro Arg Glu Val Gln Met Asp Phe Val Arg Ile Leu Val Thr
          85           90           95
Pro Gly Tyr Cys Asn Leu Thr Thr Val His Asn Thr Pro Tyr Cys Leu
          100          105          110
Ala Met Glu Glu Ser Gln Trp Ser Cys Ile Trp
          115          120

```

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 123 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

```

Ala Ile Gly Asn Glu Asp Cys Thr Pro Trp Thr Ser Thr Leu Ile Thr
1           5           10
Pro Leu Pro Ser Cys Arg Asn Tyr Val Glu Glu Gln Ala Cys Arg Ile
          20           25           30
Glu Met Pro Gly Pro Pro Tyr Leu Ala Lys Gln Glu Cys Cys Glu Gln
          35           40           45
Leu Ala Asn Ile Pro Gln Gln Cys Arg Cys Gln Ala Leu Arg Tyr Phe
          50           55           60
Met Gly Pro Lys Ser Arg Pro Asp Gln Ser Gly Leu Met Glu Leu Pro
65           70           75           80
Gly Cys Pro Arg Glu Val Gln Met Asn Phe Val Pro Ile Leu Val Thr
          85           90           95

```

Pro Gly Tyr Cys Asn Leu Thr Thr Val His Asn Thr Pro Tyr Cys Leu
100 105 110

Gly Met Glu Glu Ser Gln Trp Ser Cys Ile Trp
115 120

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 377 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

AAGCTTACGA	TGTTGCTGGC	GGGGGTGGTG	CTCAACAATG	CCCTGTAGAG	ACAAAGCTAA	60
ATTCATGCAG	GAATTACCTG	CTAGATCGAT	GCTCAACGAT	GAAGGATTTT	CCGGTCACCT	120
GGCGTTGGTG	GAAATGGTGG	AAGGGAGGTT	GTCAAGAGCT	CCTTGGGGAG	TGTTGCAGTC	180
GGCTCGGCCA	AATGCCACCG	CAATGCCGCT	GCAACATCAT	CCAGGGGTCA	ATCCAAGGCG	240
ATCTCGGTGG	CATCTTCGGA	TTTCAGCGTG	ATCGGGCAAG	CAAAGTGATA	CAAGAAGCCA	300
AGAACCTGCC	GCCCAGGTGC	AACCAGGGCC	CTCCCTGCAA	CATCCCCGGC	ACTATTGGCT	360
ATTACTGGTG	CATATGG					377

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 125 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Ala Tyr Asp Val Ala Gly Gly Gly Gly Ala Gln Gln Cys Pro Val Glu
1 5 10 15
Thr Lys Leu Asn Ser Cys Arg Asn Tyr Leu Leu Asp Arg Cys Ser Thr
20 25 30
Met Lys Asp Phe Pro Val Thr Trp Arg Trp Trp Lys Trp Trp Lys Gly
35 40 45

Gly Cys Gln Glu Leu Leu Gly Glu Cys Cys Ser Arg Leu Gly Gln Met
50 55 60

Pro Pro Gln Cys Arg Cys Asn Ile Ile Gln Gly Ser Ile Gln Gly Asp
65 70 75 80

Leu Gly Gly Ile Phe Gly Phe Gln Arg Asp Arg Ala Ser Lys Val Ile
85 90 95

Gln Glu Ala Lys Asn Leu Pro Pro Arg Cys Asn Gln Gly Pro Pro Cys
100 105 110

Asn Ile Pro Gly Thr Ile Gly Tyr Tyr Trp Cys Ile Trp
115 120 125

CLAIMS:

1. A method for producing a modified glutenin or seed-storage protein, the method comprising adding to the protein an exogenous amino acid domain which confers to the modified protein the ability to bind a ligand or other macromolecule, wherein the modified protein has an ability to incorporate into gluten.
2. The method according to claim 1 wherein the domain includes one or more cysteine residues.
3. The method according to claim 1 wherein one or more cysteine residues are incorporated at one or both ends of the amino acid sequence of the protein.
4. The method according to claim 1 wherein the modified protein further comprises one or more exogenous amino acid residues which confer to the protein an enhanced ability to incorporate into gluten.
5. The method according to claim 1 wherein the ligand or other macromolecule to which the domain binds is selected from the group consisting of lipid, and starch.
6. The method according to claim 5 wherein the domain capable of binding lipid is derived from barley oleosin gene or the lipid-binding regions of wheat CM16 protein.
7. The method according to claim 5 wherein the domain capable of binding starch is derived from glucoamylase from *Aspergillus niger*.
8. The method according to any one of claims 1 to 7 wherein the glutenin or seed-storage protein is selected from the group consisting of low molecular weight glutenins, high molecular weight glutenins, gliadins, puroindolines, grain softness proteins, friabilins, and Chloroform/Methanol-soluble proteins.
9. The method according to claim 8 wherein the glutenin or seed-storage protein is C hordein from barley.
10. A modified glutenin or seed-storage protein having an ability to incorporate into gluten and having an exogenous amino acid domain inserted therein which confers to the modified protein the ability to bind a ligand or other macromolecule produced by the method according to any one of claims 1 to 9.

11. A modified glutenin or seed-storage protein having an ability to incorporate into gluten and having an exogenous amino acid domain inserted therein which confers to the modified protein the ability to bind a ligand or other macromolecule.
- 5 12. A modified glutenin or seed-storage protein according to claim 11 wherein the ligand or other macromolecule to which the domain binds is selected from the group consisting of lipid, and starch.
- 10 13. A modified glutenin or seed-storage protein according to claim 12 wherein the domain capable of binding lipid is derived from barley oleosin gene or the lipid-binding regions of wheat CM16 protein.
14. A modified glutenin or seed-storage protein according to claim 12 wherein the domain capable of binding starch is derived from glucoamylase from *Aspergillus niger*.
- 15 15. A modified glutenin or seed-storage protein according to any one of claims 10 to 14 wherein the glutenin or seed-storage protein is selected from the group consisting of low molecular weight glutenins, high molecular weight glutenins, gliadins, puroindolines, grain softness proteins, friabilins, and Chloroform/Methanol-soluble proteins.
- 20 16. A modified glutenin or seed-storage protein selected from the group consisting of ANG/SBD/Cys7Cys236, ANG/OHBD/Cys7Cys236, and ANG/CM16/Cys7Cys236, as hereinbefore defined.
- 25 17. An isolated nucleic acid molecule encoding a modified glutenin or seed-storage protein according to any one of claims 10 to 16.
18. A cell containing an isolated nucleic acid molecule according to claim 17 such that on expression of the nucleic acid molecule, the cell produces the modified glutenin or seed-storage protein.
19. The cell according to claim 18 selected from the group consisting of bacteria, yeast, plant, insect, and mammal.
- 30 20. The cell according to claim 19 being *Escherichia coli*.
21. The cell according to claim 19 being *Pichia* sp. or *Saccharomyces cerevisiae*.
22. The cell according to claim 19 being a recombinant wheat cell.
23. Use of a modified glutenin or seed-storage protein according to any one of claims 10 to 16 in the preparation of a food product.

PCT/AU99/00563
Received 1 June 2000

45

24. The use according to claim 23 wherein the food product is selected from the group consisting of leavened or unleavened breads, pasta, noodles, breakfast cereals, snack foods, cakes, pastries, and foods containing flour-based sauces.

5 25. Use of a modified glutenin or seed-storage protein according to any one of claims 10 to 16 in the preparation of a non-food product.

26. The use according to claim 25 wherein the non-food product is selected from the group consisting of films, coatings, adhesives, building materials, and packaging materials.

10 27. Use of a grain or part of a grain containing a modified glutenin or seed-storage protein according to any one of claims 10 to 16 in the preparation of a food product.

AMENDED SHEET
IPEA/AU

PCT/AU99/00563
Received 25 July 2000

16

ABSTRACT

A method for producing a modified glutenin or seed-storage protein, the method comprising adding to the protein an exogenous amino acid domain which confers to the modified protein the ability to bind a ligand or other macromolecule, wherein the modified protein has an ability to incorporate into gluten, modified glutenin or seed-storage proteins, and uses thereof.

PCT/AU99/00563

AMENDED SHEET
(P. 2/2)

1 / 17

5' ... ATAGAATAC A GCATGC TCC CGGCCG CCATGG CCGCGG GATTGTC ATG AGG CAA CTA AAC CCT TGC AGC.....
 SP6 Promoter<| SphI EagI NcoI SacII BspHI M R Q L N P C S

ANG SEQUENCE.....GTC CCC CAA CAA GCT TCA TGC ATA TCG AGT ATG GTC TAG GGATCC
 HindIII NdeI BamHI
 V P Q Q A S C I W S M V ***

GGGTACC GAGCTC GAATTC GCCCTATA... 3'
 KpnI SacI EcoRI |> T7 Promoter

Figure 1

Figure 1

oligonucleotide 3

15

31

53

9

0

51

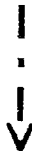
3.

691

2

1

Figure 2



3 / 17

1
ATGAGGCAACTAAACCCT**TGC**AGCCAAGAGTTGCAATCACCACAACAATCATATCTGCCG
M R Q L N P **Cys** S Q E L Q S P Q Q S Y L Q

61
CAGCCATATCCACAAAACCCATATCTACCGCAAAAACCATTTCCAGTGCAGCAACCGTTT
Q P Y P Q N P Y L P Q K P F P V Q Q P F

121
CACACACCCCAACAATATTTCCCCTATCTACCAGAGGAATTGTTTCCCAATATCAAATA
H T P Q Q Y F P Y L P E E L F P Q Y Q I

181
CCAACCCCCCTACAACCACAACAACCATTTCCCCCAACAACCACAACAACCTCTTCCTCGG
P T P L Q P Q Q P F P Q Q P Q Q P L P R

241
CCCCAACAACCATTTCCCCTGGCAACCACAACAACCATTTCCCCAGCCCCAAGAACCAATT
P Q Q P F P W Q P Q Q P F P Q P Q E P I

301
CCCCAGCAACCACAACAACCATTTCCCACAGCAACCACAACAACCATTTCCCACAGCAACCA
P Q Q P Q Q P F P Q Q P Q Q P F P Q Q P

361
CAACAAATAATTTTCCAGCAACCCCAACAATCATACCTGTGCAACCTCAACAGCCATTT
Q Q I I F Q Q P Q Q S Y P V Q P Q Q P F

421 477
CCTCAACAACCTCAACCAGTCCCCCAACAA GCT TCA **TGC**ATATGGAGTATGGTCTAG
P Q Q P Q P V P Q Q A S **Cys** I W S M V ***

Figure 3

4 / 17

HindIII 1 54
AAGCTTCTACCACTCCCACCGCCGTGGCTGTGACTTTTCGATCTGACAGCTACCACCACCTAC
A S T T P T A V A V T F D L T A T T T Y

114
GGCGAGAACATCTACCTGGTCGGATCGATCTCTCAGCTGGGTGACTGGGAAACCAGCGAC
G E N I Y L V G S I S Q L G D W E T S D

174
GGCATAGCTCTGAGTGCTGACAAGTACACTTCCAGCGACCCGCTCTGGTATGTCACTGTG
G I A L S A D K Y T S S D P L W Y V T V

234
ACTCTGCCGGCTGGTGAGTCGTTTGAGTACAAGTTTATCCGCATTGAGAGCGATGACTCC
T L P A G E S F E Y K F I R I E S D D S

294
GTGGAGTGGGAGAGTGATCCCAACCGAGAATACACCGTTCCTCAGGCGTGCGGAACGTCG
V E W E S D P N R E Y T V P Q A C G T S

321 NdeI
ACCGCGACGGTGACTGACACCTGGCGGGTGCATATGG
T A T V T D T W R C I W

Figure 4

HindIII

57

AAGCTTTCGGCAATGAAGATTGCACCCCATGGATGAGTACTCTGATCACTCCACTCCCAAGC

A I G N E D C T P W M S T L I T P L P S
CM17. T

117

TGCCGTGACTATGTGGAACAACAAGCATGTCGCATCGAAACGCCCGGGTCGCCGTACCTC

C R D Y V E Q Q A C R I E T P G S P Y L
. . N . . E M . . P . .

177

GCCAAGCAGCAGTGCTGTGGGGAGCTTGCAAACATTCCGCAGCAGTGCCGATGCCAGGCG

A K Q Q C C G E L A N I P Q Q C R C Q A
. . . E . . E Q

237

CTGCGCTACTTCATGGGGCCGAAGTCTCGTCCGGATCAGAGCGGCCTCATGGAAGTCCCC

L R Y F M G P K S R P D Q S G L M E L P
.

297

GGATGCCCTAGGGAGGTGCAGATGGACTTCGTGAGGATACTCGTCACGCCGGGGTACTGC

G C P R E V Q M D F V R I L V T P G Y C
. N . . P

354

AACTTGACGACCGTTCACAACACTCCGTACTGCCTCGCTATGGAGGAGTCTCAGTGG

N L T T V H N T P Y C L A M E E S Q W
. G

357 NdeI

AGCTGCATATGG

S C I W

Figure 5

6 / 17

HindIII 57
AAGCTTTACGATGTTGCTGGCGGGGGTGGTGTCTCAACAATGCCCTGTAGAGACAAAGCTAAAT
A Y D V A G G G G A Q Q C P V E T K L N

117
TCATGCAGGAATTACCTGCTAGATCGATGCTCAACGATGAAGGATTTCCCGGTCACCTGG
S C R N Y L L D R C S T M K D F P V T W

177
CGTTGGTGGAAATGGTGGAAAGGGAGGTTGTCAAGAGCTCCTTGGGGAGTGTTCAGTCGG
R W W K W W K G G C Q E L L G E C C S R

237
CTCGGCCAAATGCCACCGCAATGCCGCTGCAACATCATCCAGGGGTCAATCCAAGGCGAT
L G Q M P P Q C R C N I I Q G S I Q G D

297
CTCGGTGGCATCTTCGGATTTTCAGCGTGATCGGGCAAGCAAAGTGATACAAGAAGCCAAG
L G G I F G F Q R D R A S K V I Q E A K

300
AACCTGCCGCCCAGGTGCAACCAGGGCCCTCCCTGCAACATCCCCGGCACTATTGGCTAT
N L P P R C N Q G P P C N I P G T I G Y

363 NdeI
TACTGGTGCATATGG
Y W C I W

Figure 6

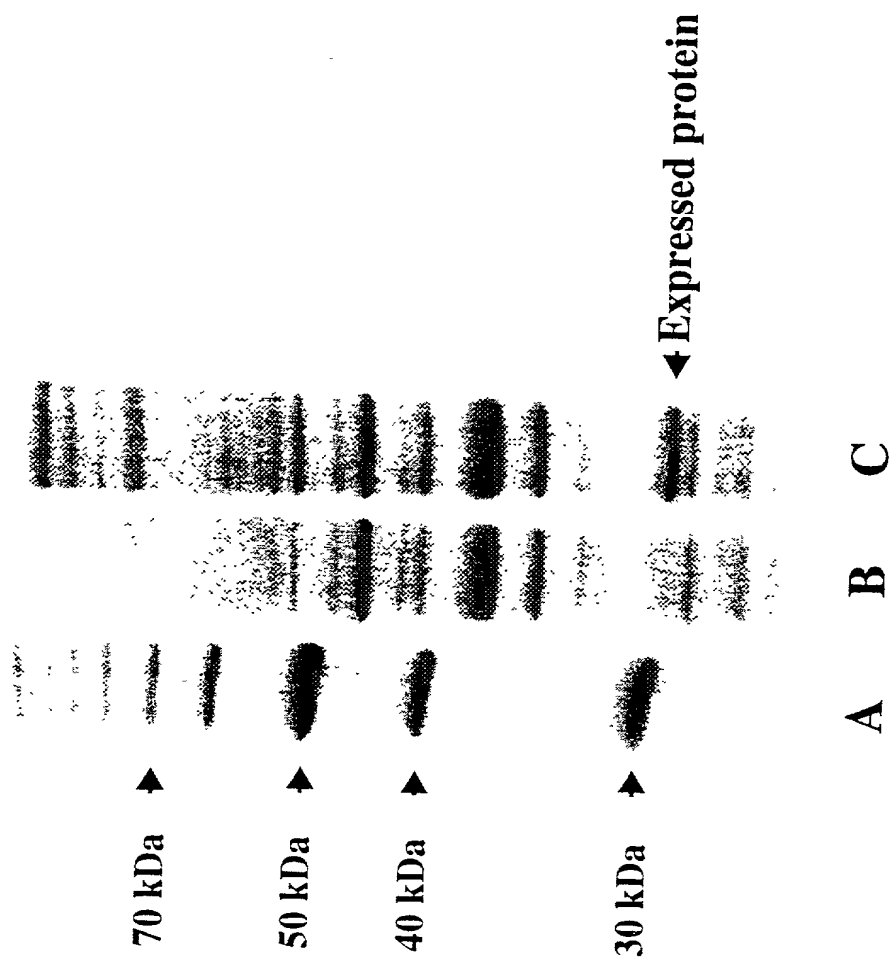


Figure 7

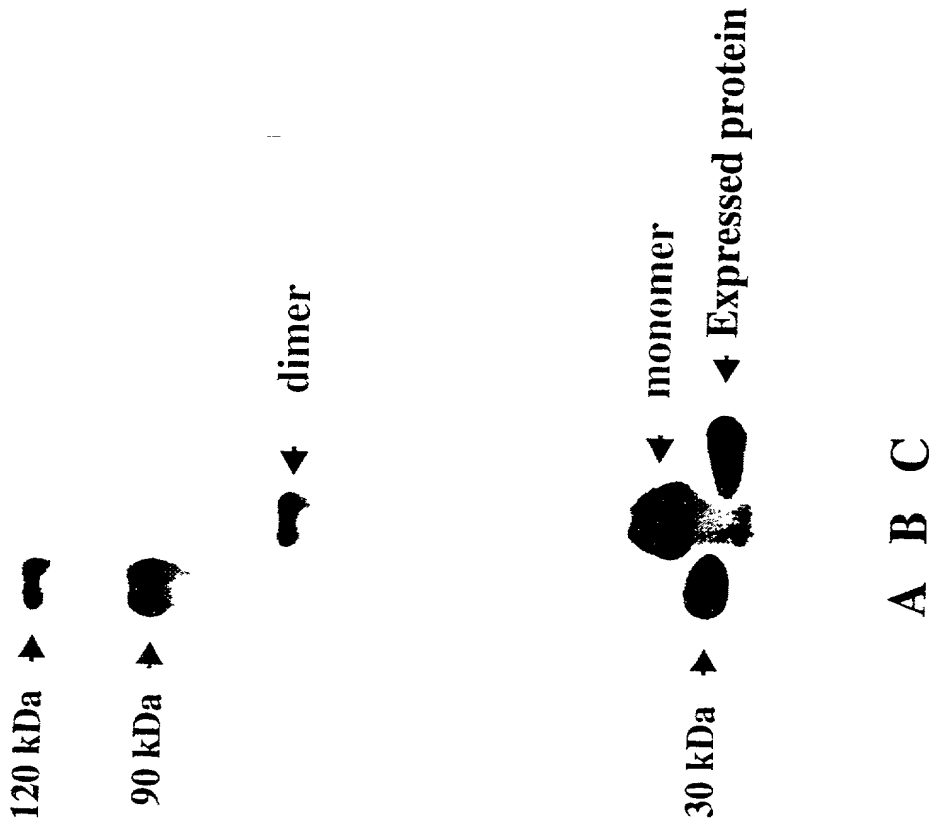


Figure 8

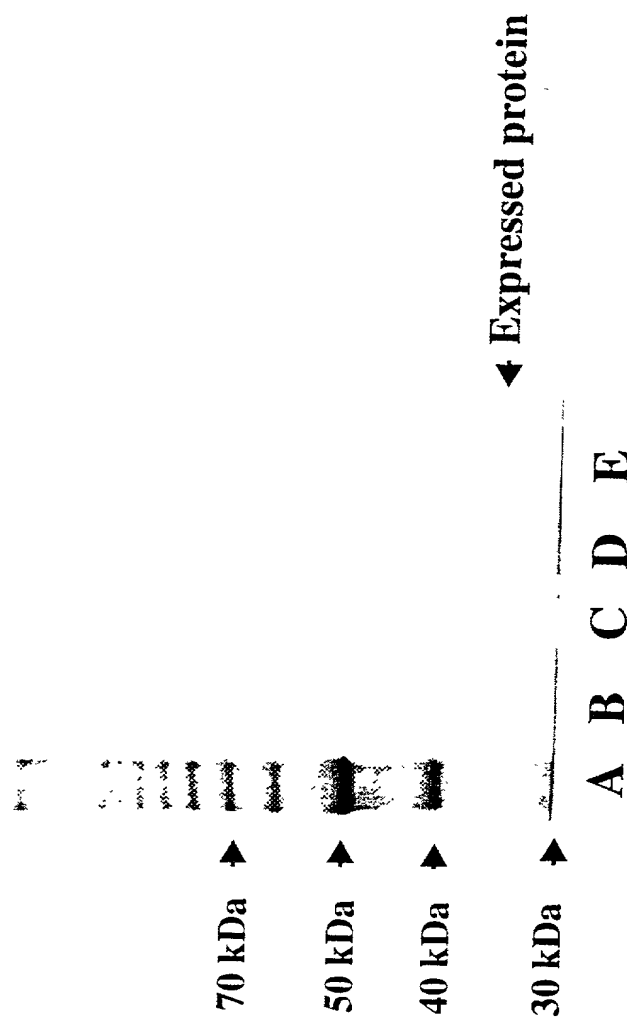


Figure 9

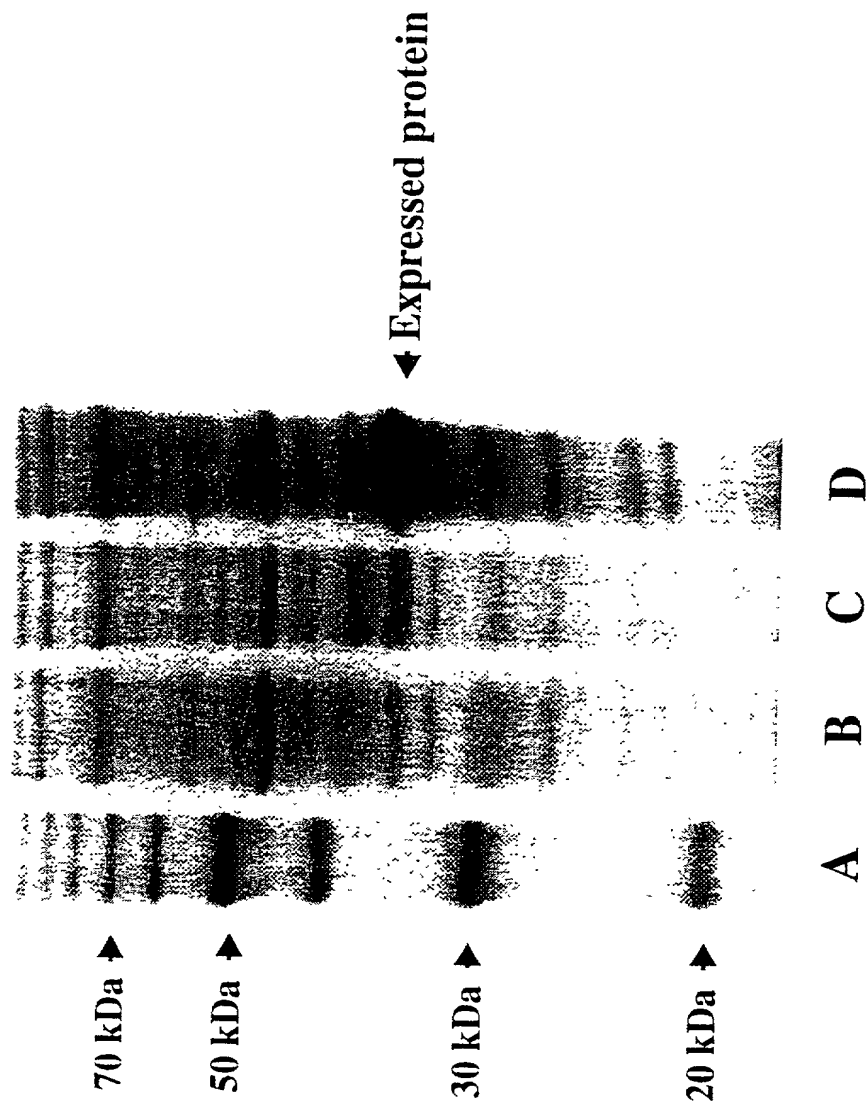


Figure 10

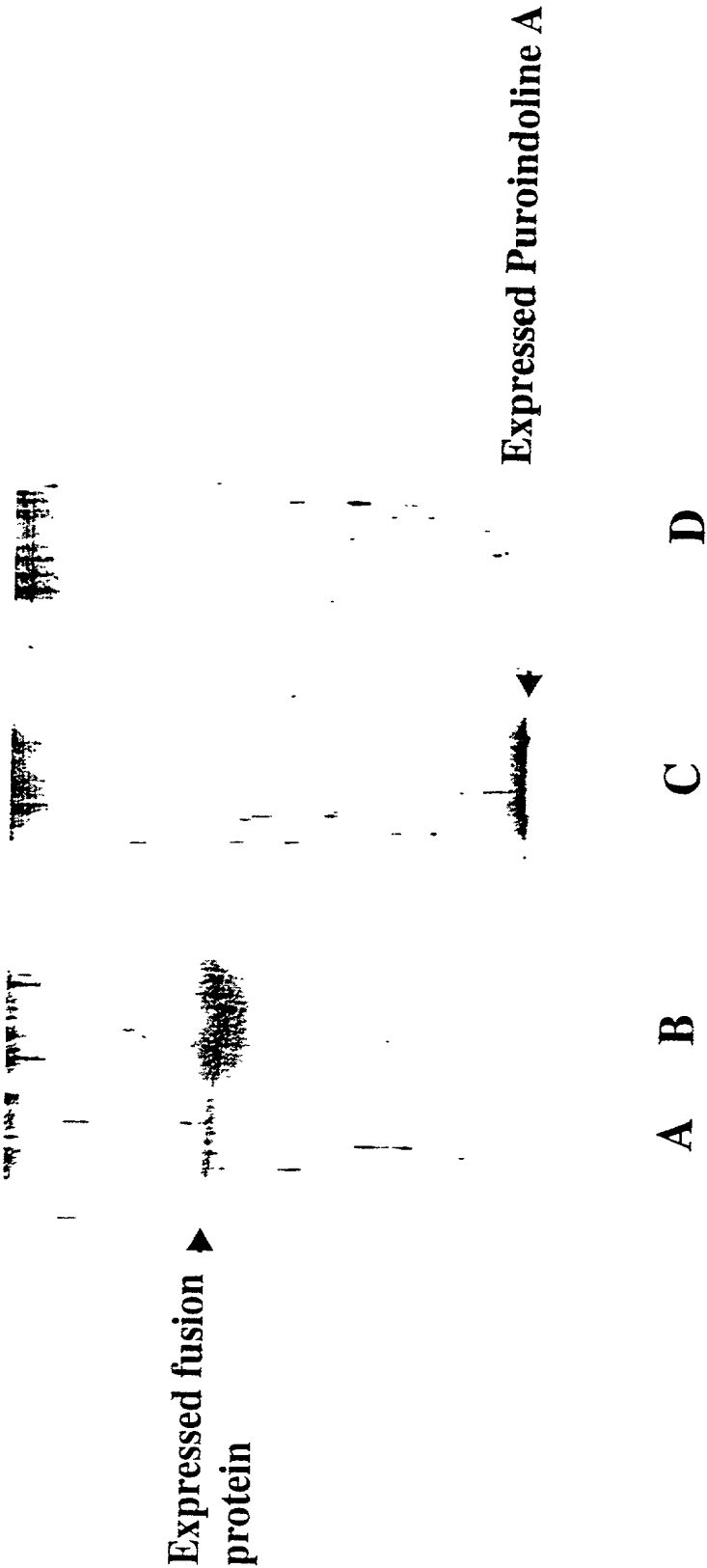


Figure 11

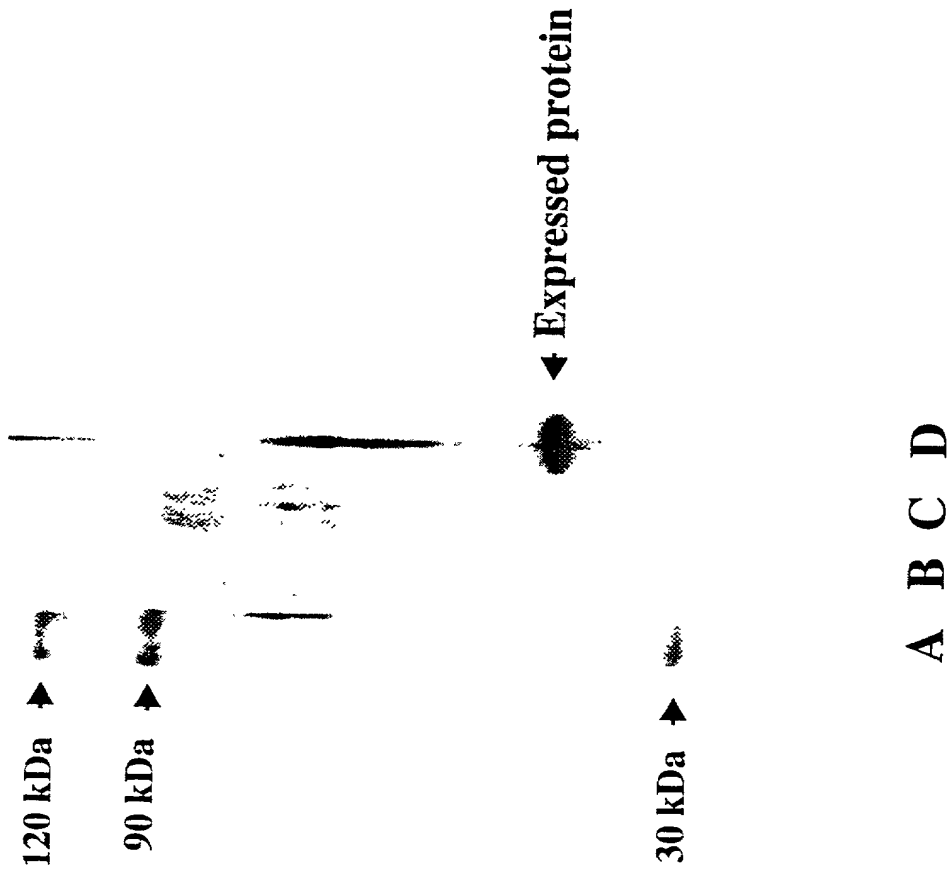


Figure 12

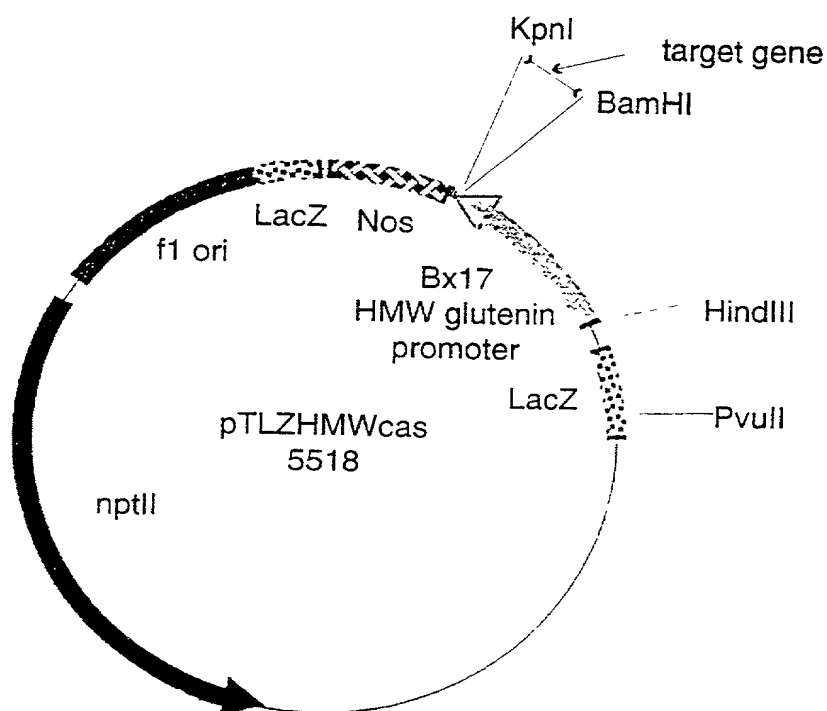


Figure 13

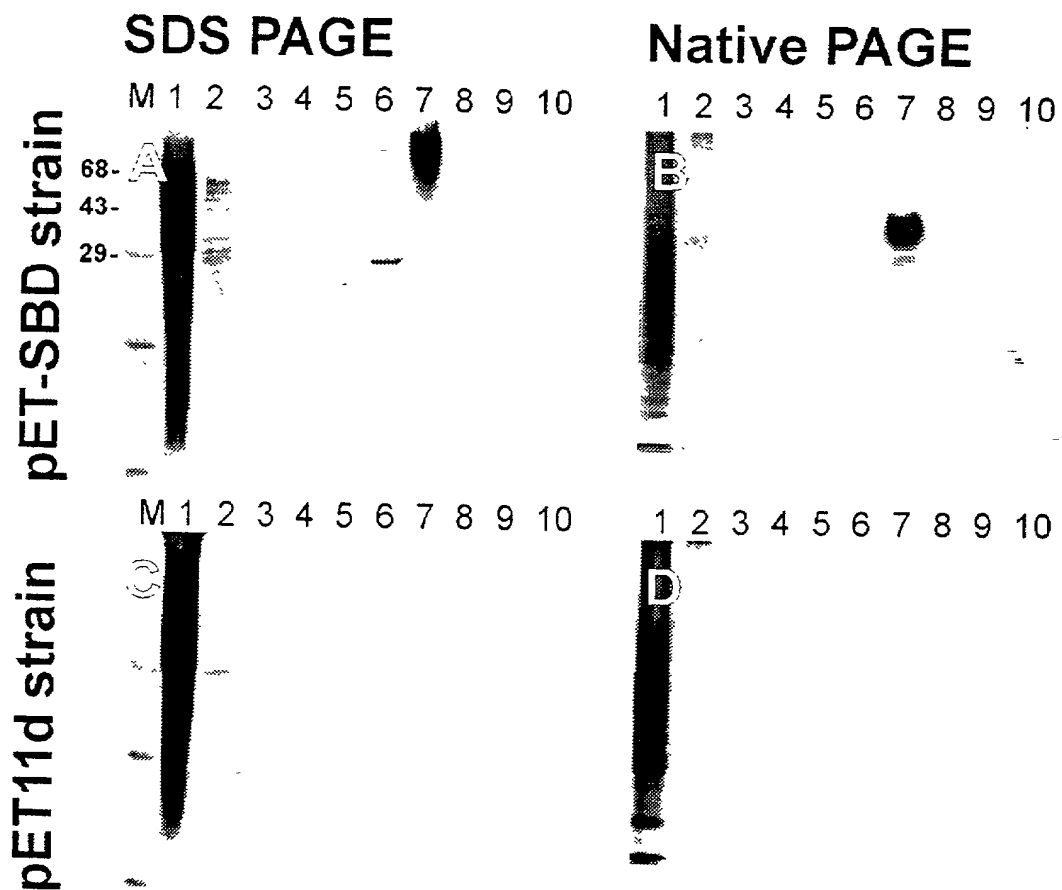
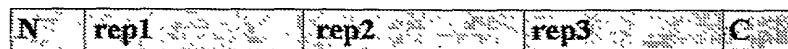
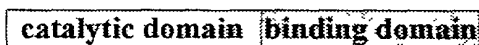


Figure 14

ANGCys7Cys236



binding domain in another protein



ANG/insert/Cys7Cys236

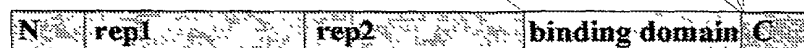


Figure 15

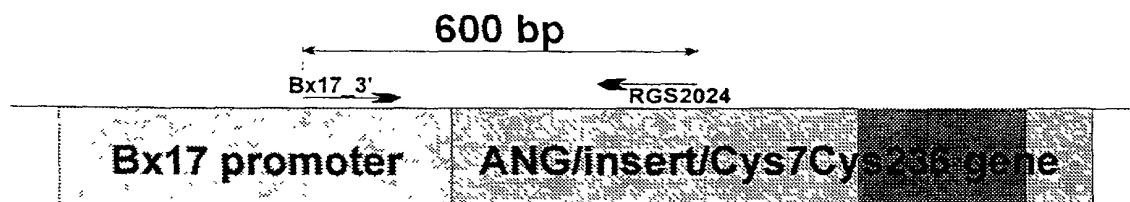


Figure 16

16 / 17

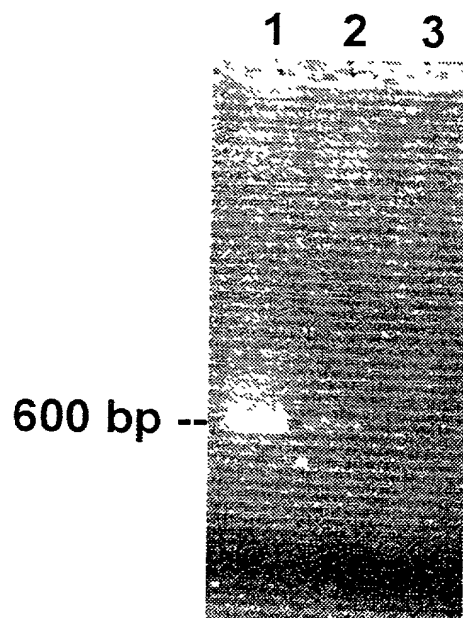


Figure 17

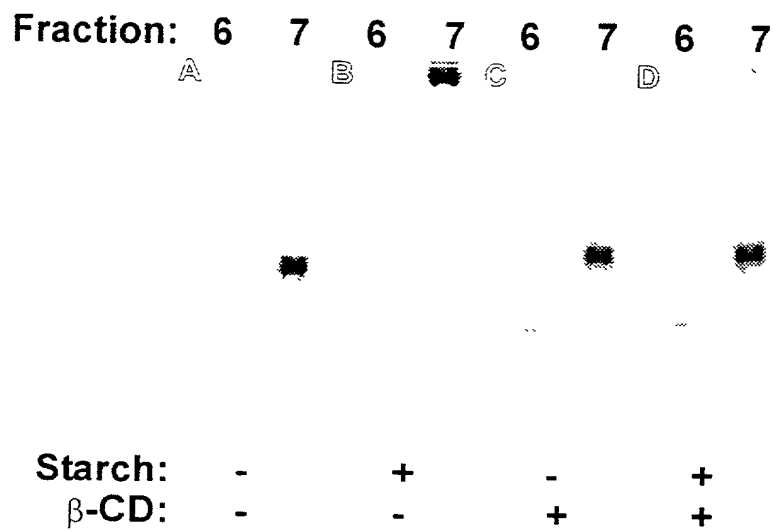


Figure 16

17 / 17

	M	1	2	3	4	5	6
oxidised	markers	+	+	+	+	-	-
reduced		-	-	+	+	+	+
β -CD		+	-	+	-	+	-



Figure 19

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled- MODIFIED PROTEINS the specification of which

☐

is attached hereto

☒

was filed on: July 12, 1999
as Application Serial No.: PCT/AU99/000563

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):

Priority Claimed

<u>PP 4604</u> (Number)	<u>Australia</u> (Country)	<u>10 July 1998</u> (Day/Month/Year Filed)	<input checked="" type="checkbox"/> <input type="checkbox"/>
			Yes No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)
--------------------------	---------------	--

I hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Harold C. Hohbach, Reg. No. 17,757; Aldo J. Test, Reg. No. 18,048; Donald N. MacIntosh, Reg. No. 20,316; Edward S. Wright, Reg. No. 24,903; David J. Brezner, Reg. No. 24,774; Robert B. Chickering, Reg. No. 24,286; Richard F. Trecartin, Reg. No. 31,801; Steven F. Caserza, Reg. No. 29,780; Michael A. Kaufman, Reg. No. 32,988; Edward N. Bachand, Reg. No. 37,085; R. Michael Ananian, Reg. No. 35,050; Robin M. Silva, Reg. No. 38,304; Maria S. Swiatek, Reg. No. 37,244; Todd A. Lorenz, Reg. No. 39,754, provided that if any one of said attorneys ceases being affiliated with the law firm of Flehr Hohbach Test Albritton & Herbert LLP as partner, employee or of counsel, such attorney's appointment as attorney and all powers derived therefrom shall terminate on the date such attorney ceases being so affiliated.

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File No. A-70233/RFT

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Date:

11-07-01

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Australia

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Inventor's signature:

Date:

13-8-01

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Citizenship:

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Laszlo TAMAS

Inventor's signature:

Date:

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(ii) TITLE OF INVENTION: Modified Proteins

(iii) NUMBER OF SEQUENCES: 26

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GTCATGAGGC AACTAAACCC TTGCAGCCAA GAGTTGCAAT C

41

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 51 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGATCCCTAG ACCATACTCC ATATGCATGA AGCTTGTTGG GGGACTGGTT G 51

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CAAGCTTGTA CCACTCCCAC CGCC 24

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCATATGCAC CGCCAGGTGT CAGTCAC 27

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTCGGCAATG AAGATTGCAC C 21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
TCCAAGTGGC TTCTCCTCTT GGCC

24

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:
GGATCCCTAG CTCCACTGAG ACTC

24

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 33 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
TGCGCTCAAG CTTTAGGCAA TGAAGATTGC ACC

33

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
CATACTCCAT ATGCAGCTCC ACTGAGACTC

30

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CAAGCTTACG ATGTTGCTGG CGGG

24

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCATATGCAC CAGTAATAGC CAATAGTGC

29

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CAACCATGTC CTGAACCTTC ACC

23

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TGGCTGTTGA GGTTGCAC

18

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 140 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

```
ATAGAATACA GCATGCTCCC GGCCGCCATG GCCGCGGGAT TGTCATGAGG CAACTAAACC   60
CTTGCAGCGT CCCCCAACAA GCTTCATGCA TATGGAGTAT GGTCTAGGGA TCCGGGTACC 120
GAGCTCGAAT TCGCCCTATA                                     140
```

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

```
Met Arg Gln Leu Asn Pro Cys Ser Val Pro Gln Gln Ala Ser Cys Ile
 1             5             10             15
Trp Ser Met Val
                20
```

(2) INFORMATION FOR SEQ ID NO: 16:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 243 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```
CAAGCTTTGA AAGCCGCTAC TGCGACAGCA GCTGGATCAT TGCTTGTGCT ATCCGGACTA   60
ATACTAGCTG GCACAGTCAT AGCACTCACA GTGGCCACAC CAGTGCTAGT CATATTTAGC 120
CCAGTGCTAG TGCCAGCGGC CATAGCCCTA GCGCTAATGT CAGCAGGCTT TGTCACGTCA 180
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GGCGGGCTGG GCGTGGCTGC GCTGAGCTCC TTTAGTGTGT TAGCCAATAC TGCCTGCATA 240
TGG 243

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 81 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Gln Ala Leu Lys Ala Ala Thr Ala Thr Ala Ala Gly Ser Leu Leu Val
1 5 10 15
Leu Ser Gly Leu Ile Leu Ala Gly Thr Val Ile Ala Leu Thr Val Ala
20 25 30
Thr Pro Val Leu Val Ile Phe Ser Pro Val Leu Val Pro Ala Ala Ile
35 40 45
Ala Leu Ala Leu Met Ser Ala Gly Phe Val Thr Ser Gly Gly Leu Gly
50 55 60
Val Ala Ala Leu Ser Ser Phe Ser Val Leu Ala Asn Thr Ala Cys Ile
65 70 75 80
Trp

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

ATGAGGCAAC TAAACCCTTG CAGCCAAGAG TTGCAATCAC CACAACAATC ATATCTGCCG 60
CAGCCATATC CACAAAACCC ATATCTACCG CAAAAACCAT TTCCAGTGCA GCAACCGTTT 120
CACACACCCC AACAATATTT CCCCTATCTA CCAGAGGAAT TGTTTCCCCA ATATCAAATA 180
CCAACCCCCC TACAACCACA ACAACCATT CCCCCAACAAC CACAACAACC TCTTCCTCGG 240
CCCCAACAAC CATTCCCCTG GCAACCACAA CAACCATTTC CCCAGCCCCA AGAACCAATT 300

CCCCAACAAC CATTCCCCTG GCAACCACAA CAACCATTTC CCCAGCCCCA AGAACCAATT 360
 CAACAAATAA TTTTCCAGCA ACCCCAACAA TCATACCCTG TGCAACCTCA ACAGCCATTT 420
 CCTCAACAAC CTCAACCAGT CCCCCAACAA GCTTCATGCA TATGGAGTAT GGTCTAG 477

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 158 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Met	Arg	Gln	Leu	Asn	Pro	Cys	Ser	Gln	Glu	Leu	Gln	Ser	Pro	Gln	Gln	1	5	10	15
Ser	Tyr	Leu	Gln	Gln	Pro	Tyr	Pro	Gln	Asn	Pro	Tyr	Leu	Pro	Gln	Lys	20	25	30	
Pro	Phe	Pro	Val	Gln	Gln	Pro	Phe	His	Thr	Pro	Gln	Gln	Tyr	Phe	Pro	35	40	45	
Tyr	Leu	Pro	Glu	Glu	Leu	Phe	Pro	Gln	Tyr	Gln	Ile	Pro	Thr	Pro	Leu	50	55	60	
Gln	Pro	Gln	Gln	Pro	Phe	Pro	Gln	Gln	Pro	Gln	Gln	Pro	Leu	Pro	Arg	65	70	75	80
Pro	Gln	Gln	Pro	Phe	Pro	Trp	Gln	Pro	Gln	Gln	Pro	Phe	Pro	Gln	Pro	85	90	95	
Gln	Glu	Pro	Ile	Pro	Gln	Gln	Pro	Gln	Gln	Pro	Phe	Pro	Gln	Gln	Pro	100	105	110	
Gln	Gln	Pro	Phe	Pro	Gln	Gln	Pro	Gln	Gln	Ile	Ile	Phe	Gln	Gln	Pro	115	120	125	
Gln	Gln	Ser	Tyr	Pro	Val	Gln	Pro	Gln	Gln	Pro	Phe	Pro	Gln	Gln	Pro	130	135	140	
Gln	Pro	Val	Pro	Gln	Gln	Ala	Ser	Cys	Ile	Trp	Ser	Met	Val	145	150	155			

(2) INFORMATION FOR SEQ ID NO: 20:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 338 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

```

AAGCTTCTAC CACTCCCACC GCCGTGGCTG TGACTTTCGA TCTGACAGCT ACCACCACCT  60
ACGGCGAGAA CATCTACCTG GTCGGATCGA TCTCTCAGCT GGGTGACTGG GAAACCAGCG 120
ACGGCATAGC TCTGAGTGCT GACAAGTACA CTTCCAGCGA CCGCTCTGG TATGTCACTG 180
TGACTCTGCC GGCTGGTGAG TCGTTTGAGT ACAAGTTTAT CCGCATTGAG AGCGATGACT 240
CCGTGGAGTG GGAGAGTGAT CCCAACCGAG AATACACCGT TCCTCAGGCG TGCGGAACGT 300
CGACCGCGAC GGTGACTGAC ACCTGGCGGT GCATATGG                               338

```

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 112 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

```

Ala Ser Thr Thr Pro Thr Ala Val Ala Val Thr Phe Asp Leu Thr Ala
1           5           10           15
Thr Thr Thr Tyr Gly Glu Asn Ile Tyr Leu Val Gly Ser Ile Ser Gln
20        25        30
Leu Gly Asp Trp Glu Thr Ser Asp Gly Ile Ala Leu Ser Ala Asp Lys
35        40        45
Tyr Thr Ser Ser Asp Pro Leu Trp Tyr Val Thr Val Thr Leu Pro Ala
50        55        60
Gly Glu Ser Phe Glu Tyr Lys Phe Ile Arg Ile Glu Ser Asp Asp Ser
65        70        75        80
Val Glu Trp Glu Ser Asp Pro Asn Arg Glu Tyr Thr Val Pro Gln Ala
85        90        95
Cys Gly Thr Ser Thr Ala Thr Val Thr Asp Thr Trp Arg Cys Ile Trp
100       105       110

```

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 371 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

```

AAGCTTTCGG CAATGAAGAT TGCACCCCAT GGATGAGTAC TCTGATCACT CCACTCCCAA 60
GCTGCCGTGA CTATGTGGAA CAACAAGCAT GTCGCATCGA AACGCCCGGG TCGCCGTACC 120
TCGCCAAGCA GCAGTGCTGT GGGGAGCTTG CAAACATTCC GCAGCAGTGC CGATGCCAGG 180
CGCTGCGCTA CTTTCATGGGG CCGAAGTCTC GTCCGGATCA GAGCGGCCTC ATGGAAGTCC 240
CCGGATGCCC TAGGGAGGTG CAGATGGACT TCGTGAGGAT ACTCGTCACG CCGGGGTACT 300
GCAACTTGAC GACCGTTCAC AACACTCCGT ACTGCCTCGC TATGGAGGAG TCTCAGTGGA 360
GCTGCATATG G 371

```

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 123 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

```

Ala Ile Gly Asn Glu Asp Cys Thr Pro Trp Met Ser Thr Leu Ile Thr
1           5           10           15
Pro Leu Pro Ser Cys Arg Asp Tyr Val Glu Gln Gln Ala Cys Arg Ile
20          25          30
Glu Thr Pro Gly Ser Pro Tyr Leu Ala Lys Gln Gln Cys Cys Gly Glu
35          40          45
Leu Ala Asn Ile Pro Gln Gln Cys Arg Cys Gln Ala Leu Arg Tyr Phe
50          55          60
Met Gly Pro Lys Ser Arg Pro Asp Gln Ser Gly Leu Met Glu Leu Pro
65          70          75          80
Gly Cys Pro Arg Glu Val Gln Met Asp Phe Val Arg Ile Leu Val Thr
85          90          95
Pro Gly Tyr Cys Asn Leu Thr Thr Val His Asn Thr Pro Tyr Cys Leu
100         105         110
Ala Met Glu Glu Ser Gln Trp Ser Cys Ile Trp
115         120

```

(2) INFORMATION FOR SEQ ID NO: 24:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 123 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

```

Ala Ile Gly Asn Glu Asp Cys Thr Pro Trp Thr Ser Thr Leu Ile Thr
1             5             10             15

Pro Leu Pro Ser Cys Arg Asn Tyr Val Glu Glu Gln Ala Cys Arg Ile
                20             25             30

Glu Met Pro Gly Pro Pro Tyr Leu Ala Lys Gln Glu Cys Cys Glu Gln
            35             40             45

Leu Ala Asn Ile Pro Gln Gln Cys Arg Cys Gln Ala Leu Arg Tyr Phe
            50             55             60

Met Gly Pro Lys Ser Arg Pro Asp Gln Ser Gly Leu Met Glu Leu Pro
65             70             75             80

Gly Cys Pro Arg Glu Val Gln Met Asn Phe Val Pro Ile Leu Val Thr
            85             90             95

Pro Gly Tyr Cys Asn Leu Thr Thr Val His Asn Thr Pro Tyr Cys Leu
            100            105            110

Gly Met Glu Glu Ser Gln Trp Ser Cys Ile Trp
            115            120

```

(2) INFORMATION FOR SEQ ID NO: 25:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 377 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

```

AAGCTTACGA TGTTGCTGGC GGGGGTGGTG CTCAACAATG CCCTGTAGAG ACAAAGCTAA 60
ATTCATGCAG GAATTACCTG CTAGATCGAT GCTCAACGAT GAAGGATTTC CCGGTCACCT 120
GGCGTTGGTG GAAATGGTGG AAGGGAGGTT GTCAAGAGCT CCTTGGGGAG TGTTGCAGTC 180
GGCTCGGCCA AATGCCACCG CAATGCCGCT GCAACATCAT CCAGGGGTCA ATCCAAGGCG 240
ATCTCGGTGG CATCTTCGGA TTTCAGCGTG ATCGGGCAAG CAAAGTGATA CAAGAAGCCA 300

```

AGAACCTGCC GCCCAGGTGC AACCAGGGCC CTCCCTGCAA CATCCCCGGC ACTATTGGCT 360
ATTACTGGTG CATATGG 377

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 125 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Ala	Tyr	Asp	Val	Ala	Gly	Gly	Gly	Gly	Ala	Gln	Gln	Cys	Pro	Val	Glu	
1				5					10					15		
Thr	Lys	Leu	Asn	Ser	Cys	Arg	Asn	Tyr	Leu	Leu	Asp	Arg	Cys	Ser	Thr	
			20					25					30			
Met	Lys	Asp	Phe	Pro	Val	Thr	Trp	Arg	Trp	Trp	Lys	Trp	Trp	Lys	Gly	
		35					40					45				
Gly	Cys	Gln	Glu	Leu	Leu	Gly	Glu	Cys	Cys	Ser	Arg	Leu	Gly	Gln	Met	
	50					55					60					
Pro	Pro	Gln	Cys	Arg	Cys	Asn	Ile	Ile	Gln	Gly	Ser	Ile	Gln	Gly	Asp	
65					70					75				80		
Leu	Gly	Gly	Ile	Phe	Gly	Phe	Gln	Arg	Asp	Arg	Ala	Ser	Lys	Val	Ile	
			85						90					95		
Gln	Glu	Ala	Lys	Asn	Leu	Pro	Pro	Arg	Cys	Asn	Gln	Gly	Pro	Pro	Cys	
			100					105					110			
Asn	Ile	Pro	Gly	Thr	Ile	Gly	Tyr	Tyr	Trp	Cys	Ile	Trp				
	115						120					125				